

Bond University

DOCTORAL THESIS

Mechanisms controlling ureteral motility

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Award date:
2017

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MECHANISMS CONTROLLING URETERAL MOTILITY

Submitted in total fulfilment of the requirements
of the degree of Doctor of Philosophy by
Research by

Iris Lim

[October, 2016]

ABSTRACT

Medical expulsive therapy can be administered to treat ureteral calculi by relieving ureteral colic and increasing stone passage rate. It is vital to have a greater understanding of the mechanisms controlling ureteral contraction, as this may reveal novel mechanisms and identify targets for the development of more effective pharmacological agents for medical expulsive therapy. The overall aim of this thesis was to elucidate the mechanisms controlling contractility of the ureter. Functional organ bath studies with isolated porcine ureteral tissues were used to examine key systems involved in ureteral smooth muscle contraction. The findings demonstrate that G-protein coupled receptor-mediated contractile responses of ureteral tissues in response to muscarinic receptor, α_1 -adrenoceptor and 5-HT receptor stimulation differ with age and the latter two appear to play more dominant roles in mediation of ureteral smooth muscle contraction. Muscarinic and α_1 -adrenoceptor-stimulated responses are increased in tissues from older animals compared to younger animals and vice versa in 5-HT-stimulated contractions. The 5-HT_{2A} receptor subtype appears to be responsible for 5-HT-mediated contractile responses, although, this might be more complex with age. While it was clear that this is the sole functional receptor subtype in ureteral tissues from the younger animals, the findings suggested that this might not be the case in ureteral tissues from older animals. In the study of intracellular signalling pathways, it was observed that the Rho-kinase pathway plays a vital role in mediation of contractile responses in the ureter. Additionally, the inhibitory effect of urothelium and specifically, urothelium-derived inhibitory factor on contractile responses was evident in the isolated ureter and adenosine triphosphate was identified as a likely candidate for this inhibitory mediator. Conclusively, age-related changes are apparent in every mechanism that was investigated, indicating that ageing might be an important factor to be considered in management of ureteral colic.

DECLARATION

This thesis is submitted to Bond University in fulfilment of the requirements of the degree of Doctor of Philosophy by Research.

I declare that the research presented within this thesis is a product of my own original ideas and work, and contains no material which has previously been submitted for a degree at this or any other institution, except where due acknowledgement has been made.

Iris Lim

7th October 2016

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisors, Donna Sellers and Russ Chess-Williams for their continual support and guidance in the development of my scientific skills. Donna, you always tell me I am very organised but the truth is, I learnt from the best – you! It will always be a wonder to me how you managed those meetings and teaching and still have time to constantly check on me. Russ, you are a special character that I have come to truly admire in these years and I will forever cherish our endless chats. You have both contributed so much in my PhD years, not just in research but also in my personal life and I will always be grateful. I would also like to thank all my colleagues in the Centre for Urology Research at Bond University for always being friends I can turn to when I run into any troubles in the lab and also for the weekly chats at our lab meeting on scientific and non-scientific topics.

A big thank you also goes to the kindest couple I know, Joe and Cynthia. You both are my God-sent guardian angels on the Gold Coast. The biggest thank you, of course, goes to my family. Seventh year away from home, but still as tough as ever. To my siblings, Aline, Lenice, Daryl and Sheryl, thanks for keeping me sane by being insane with me throughout these years and making me feel less nerdy than I really am! Last but definitely not least, infinite thanks to my amazing parents. Dad (Anthony), for believing in me even when I didn't and being the light that guides me to the right path. Mum (Celine), for the encouragement and nightly chats about my day despite how much more you had going on – no more complains about the 3-hour drive to the abattoir! I love you both endlessly and I dedicate this thesis to you.

PUBLICATIONS

Gold Coast Health and Medical Research Conference (GHMRC)

Lim I, Sellers D & Chess-Williams R (2013) The difference in contractile responses of isolated porcine tissues to carbachol and phenylephrine in old and young pigs. GCHMRC, Gold Coast, Australia, November 2013. Available from proceedings of Gold Coast Health and Medical Research Conference 2013 (p 106)

National Symposium on Advances in Gastrointestinal & Urogenital Research

Lim I, Sellers D & Chess-Williams R (2013) The effect of age on contractile responses of the porcine ureter to carbachol and phenylephrine. 5th National Symposium on Advances in Gastrointestinal & Urogenital Research, Melbourne, Australia, November 2013. Available from proceedings of 5th National Symposium on Advances in Gastrointestinal & Urogenital Research

Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists (ASCEPT) Annual Scientific Meeting

Lim I, Sellers D & Chess-Williams R (2013) The effect of age on contractile responses of the porcine ureter to carbachol and phenylephrine. ASCEPT, Melbourne, Australia, December 2013. Available online at <http://www.asceptasm.com/wp-content/uploads/2013/06/ASCEPT-abstracts-403-493.pdf> (Abstract No. 437)

National Symposium on Advances in Gastrointestinal & Urogenital Research

Lim I, Sellers D & Chess-Williams R (2014) The role of Rho-kinase in phenylephrine and 5-HT-induced contractile activity of the porcine ureter. 6th National Symposium on Advances in Gastrointestinal & Urogenital

Research, Gold Coast, Australia, September 2014. Available from proceedings of 6th National Symposium on Advances in Gastrointestinal & Urogenital Research (p 7)

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National Symposium on Advances in Gastrointestinal & Urogenital Research

Lim I, Sellers D & Chess-Williams R (2015) Depressed effect of ageing on contractile response of porcine distal ureter to 5-hydroxytryptamine via 5-HT_{2A} receptor subtype. 7th National Symposium on Advances in Gastrointestinal & Urogenital Research, Adelaide, Australia, October 2015. Available from proceedings of 7th National Symposium on Advances in Gastrointestinal & Urogenital Research (p 13)

International Continence Society (ICS) Annual Meeting

Lim I, Sellers D & Chess-Williams R (2015) Depressed effect of ageing on contractile response of porcine distal ureter to 5-hydroxytryptamine via 5-

HT_{2A} receptor subtype. ICS, Montreal, Canada, October 2015. Available online at <http://www.ics.org/Abstracts/Publish/241/000161.pdf>

Gold Coast Health and Medical Research Conference (GHMRC) (Best Digital Presentation Award)

Lim I, Sellers D & Chess-Williams R (2015) The role of Rho-kinase in phenylephrine and 5-HT-induced contractile activity of the porcine ureter. GCHMRC, Gold Coast, Australia, December 2015. Available from proceedings of Gold Coast Health and Medical Research Conference 2015

International Continence Society (ICS) Annual Meeting

Lim I, Sellers D & Chess-Williams R (2016) The role of rho-kinase in phenylephrine and 5-HT-induced contractile activity in the porcine ureter. ICS, Tokyo, Japan, September 2016. Available online at <http://www.ics.org/Abstracts/Publish/326/000642.pdf>

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CHAPTER 1:

General Introduction

1.1 Ureteric Calculi

Urinary stones, also known as urinary calculi, are stones or calcifications developed by the aggregation of crystalline mineral deposits in the urine to form hard-rock crystals of various sizes and shapes. This condition is known as urolithiasis (Romero et al., 2010). Ninety percent of calculi are calcium stones and the remaining 10% are non-calcium stones, including struvite, uric acid, ammonium urate, and xanthine stones (Kijvikai and de la Rosette, 2011). While calculi can be found in various parts of the urinary tract, they are commonly lodged in the ureter as ureteric calculi (Canda et al., 2007) (Figure 1.1). The human adult ureter is a tube of smooth muscle, approximately 25 – 30 cm in length, responsible for propelling urine from the kidney to the bladder and can be anatomically divided into three parts: the proximal, mid and distal ureter. The proximal ureter is the section closest to the kidney whilst the distal ureter is the lower section approaching the bladder. Studies have shown that the most common location for lodgement of ureteral calculi is the distal ureter approaching the ureterovesical junction (also termed the intravesical ureter) where the ureter enters the bladder. This region accounts for 54% of calculi requiring surgical intervention (El-Barky et al., 2014). The second most common location, accounting for 34% of calculi, is just below the pelvi-ureteric junction where the proximal ureter leaves the kidney (El-Barky et al., 2014).

Pathogenesis

Despite numerous studies aimed at understanding the pathogenesis of urinary calculi, the events that lead to stone formation are still unknown. This is partly because this disease is a polygenic and multifactorial disorder that is associated with involvement of an interrelationship between the kidney, bone and intestine (Gnessin, 2010).

However, tremendous progress has been made in recent years to delineate the exact processes that lead to calculi formation and a number of chemical theories including supersaturation, nucleation and crystal formation, crystal aggregation, crystal retention and crystal growth inhibitors are thought to be involved (Miller et al., 2007, Gnessin, 2010).

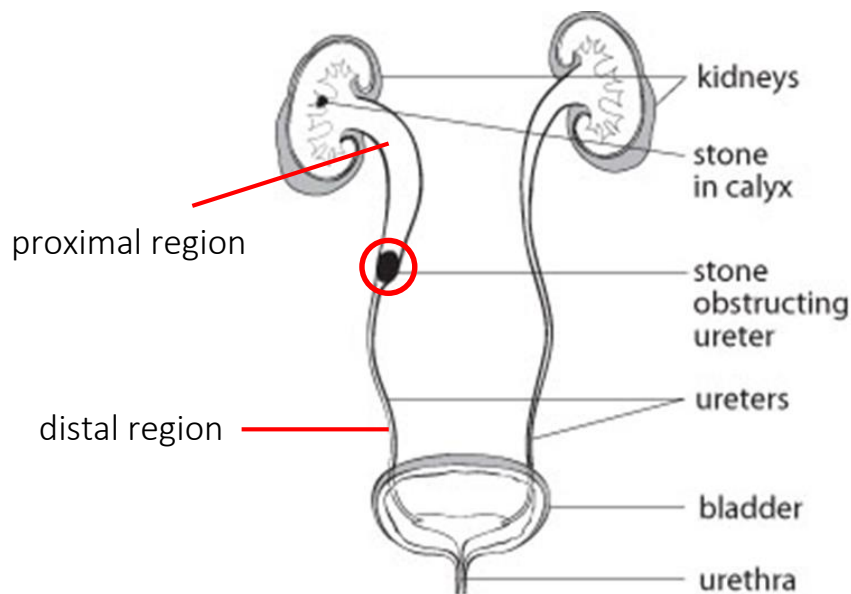


Figure 1.1 Anatomical position of ureteric stones in relation to the structures of the urinary tract (El-Barky et al., 2014).

Urinary supersaturation provides the driving force for a phase change from dissolved salt to solid phase and this occurs when the concentration of calculus-forming salt exceeds its solubility (Coe et al., 2005). It is possible to obtain supersaturation values by measuring ionic concentrations in urine tests. Calculated supersaturation values have been observed to correlate with stone composition and emphasize its importance in the pathogenesis of calculi formation (Parks et al., 1997).

Nucleation is the establishment of the smallest units of crystal formation and occurs in the presence of high supersaturation values. The two types of nucleation are homogenous and heterogenous, where homogenous nucleation occurs in a pure solution and requires a much higher supersaturation level in comparison to the latter (Khan, 1997). However, in most cases, human urine has a diverse chemical environment causing nuclei to form on structures like cellular debris, urinary crystals and existing urinary membranes. This form of nucleation is called heterogenous nucleation and requires a lower level of supersaturation values, which suggest the possibility that this type of nucleation plays a greater role in calculi formation (Gnessin, 2010). Additionally, it has been observed that most calculi are comprised of more than one crystal type, thus, also supporting the predominance of heterogenous over homogenous nucleation (Khan, 1997).

Crystal aggregation is the process where stone crystals bind to one another via chemical and electrical forces. Upon aggregation, these stone crystals are held in place and cannot be easily separated. This process is thought to have a vital role in calculi formation in the urinary tract, as a single crystal would not be large enough to be retained in the collecting system (Kok et al., 1990). In addition to this, crystal retention is also thought to be involved in calculi formation, as nucleation and crystal aggregation need to occur within the time frame of transit of urine through the nephron (Kok and Khan, 1994).

Finally, crystal growth inhibitors might play an equally important role in calculi formation as supersaturation values. It was found that urine from non-calculus-formers contained high supersaturation values but calculus formation but did not occur in these individuals. This was explained by a number of substances found in the urine including citrate, magnesium, pyrophosphate and albumin, which prevent the formation of calculi (Coe et al., 2005, Gnessin, 2010).

While these theories on the physical chemistry of calculus formation are accepted to play significant roles, it is important to keep in mind that calculus formation could be due to different mechanisms in different patients. This is highly dependent on the clinical scenario of a certain patient and therefore, it is difficult to completely specify the pathogenesis of stone formation.

Significance

Urolithiasis is a common condition and is associated with considerable morbidity and a substantial economic impact. The incidence of urolithiasis is increasing in western countries, as well as in Australia, affecting 1 in 10 people (Macneil and Bariol, 2011) and its prevalence is expected to escalate significantly in the following decades, as has been the case for obesity, diabetes and metabolic syndrome (Soucie et al., 1994, Lee et al., 2002). In addition, the number of stone treatment procedures in Australia has increased in recent years (Figure 1.2). Although the financial burden of the disease is unknown for Australia, it was estimated at approximately \$2.1 billion in 2000 in the United States, including \$971 million allocated for inpatient services which reflects an increase of 50% since 1995 (Lotan and Pearle, 2011). In 2012, urolithiasis was associated with 25,000 hospital admissions in England costing £11.6 million (Pickard et al., 2015).

Another major problem associated with urolithiasis is recurrence of disease (Yuvanc et al., 2015). It has been reported that patients have recurrence rates of 10% in the first year, 35% in the next 5 years and 50% in 10 years following the first incidence (Yuvanc et al., 2015). With recurrence, patients may require further invasive or non-invasive surgical treatment and hospitalization, which subsequently leads to increased monetary costs (Yuvanc et al., 2015).

Risk Factors

It is more likely for calculi to develop in men than in women, but the ratio has been shown to have declined from a 3:1 male-to-female predominance to now less than 1.3:1 since 1994 (Schade and Faerber, 2010). The reason for this, and the slight predominance in males, are unexplained, although one interesting proposition is that females are more likely to hydrate than males, as dehydration is a major risk factor (Schade and Faerber, 2010). While this condition can affect all age groups, the first episode commonly occurs before reaching the age of 30 and then more frequently with increasing age (Hess, 2003).

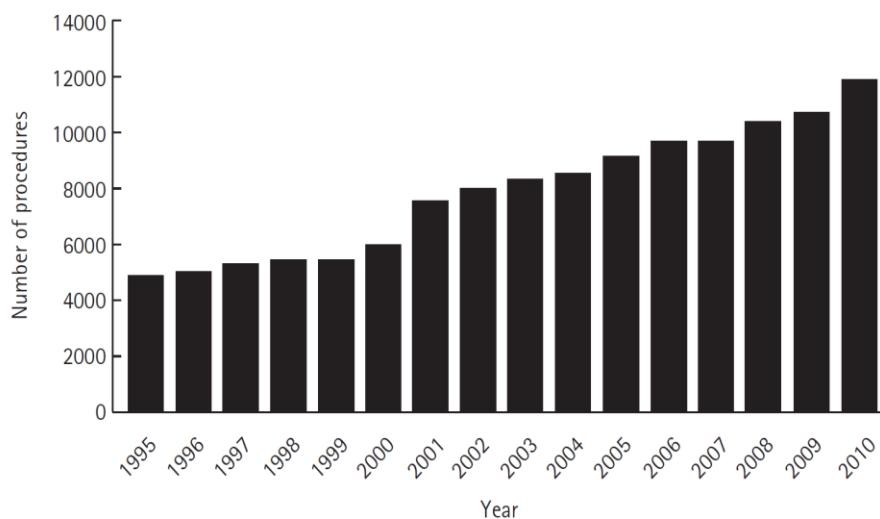


Figure 1.2 Number of procedures claimed under Medicare Australia for different stone treatment modalities between 1995 and 2010. The graph depicts a consistent increase in stone treatment procedures over the 16 years (Lee and Bariol, 2011). No more recent accreditable data on urinary calculi was obtainable.

The highest prevalence and incidence rates of ureteral calculi are observed in Caucasians, followed by Hispanics, blacks and Asians (Romero et al., 2010). Additionally, a strong family history of urolithiasis and recurrent stone formation is also a risk factor (Macneil and Bariol, 2011). Environmental conditions and eating habits, such as high sodium and protein diets, including meat, fish and poultry, accompanied with low carbohydrate intake, have also been shown to result in a higher risk of stone formation. This diet composition has been shown to reduce the body's ability to absorb calcium and therefore increases the chances of stones developing (Gottlieb, 2002).

In addition to this, diabetes mellitus, gout and obesity are also major risk factors associated with urinary stone formation (Chung et al., 2011, Nowfar et al., 2011, Marchini et al., 2013). Those affected by diabetes, specifically, type 2 diabetes, have been shown to have urine with lower pH values than non-diabetic patients. While the reason for this is still unclear, the acidity of the urine is thought to increase the probability of uric acid calculi formation (Chung et al., 2011). The build-up of uric acid in the blood that occurs in gout also increases the risk of calculi formation (Marchini et al., 2013). Additionally, obesity is associated with an increase in insulin resistance and compensatory hyperinsulinemia which may contribute to the development of calcium stones by increasing the urinary excretion of calcium (Nowfar et al., 2011). Furthermore, it was also suggested that the obese population is more inclined to consume more salt and animal protein in their diet, and thus, predisposes them to calculus formation (Nowfar et al., 2011). Chronic kidney disease has also been suggested to be a significant health problem that could lead to urinary calculi formation and various studies have linked urolithiasis with varying degrees of renal insufficiency. It has been reported that 0.8% to 17.5% of patients with chronic kidney disease present with urinary stone disease (Karthi et al., 2013). The potential causes of kidney failure in patients presenting with stones include hydronephrosis (swelling of kidney/s due to build-up of

urine), infection, diabetes mellitus, hypertension, repeated stone surgeries, diet and genetic factors (Chou et al., 2011). It is also vital to note that besides being a risk factor for stone formation, kidney disease could also be a consequence of severe urolithiasis conditions (Chou et al., 2011).

Symptoms and Diagnosis

It is observed clinically that symptom manifestation in urolithiasis patients is highly dependent on the size of the calculus. Patients with smaller calculi are thought to pass stones asymptotically in comparison to those with larger stones who could have severe symptoms (Kijvikai and de la Rosette, 2011). The most pressing symptom of urinary stones is urinary colic. Colic is an excruciating pain felt by patients at the location of the stone. The classic presentation of a ureteric colic is episodic acute colicky flank pain that radiates from the back to the groin. Physical examination usually shows a patient with ureteric colic writhing in distress, searching for a comfortable position, which is distinct from patients with peritoneal irritation who usually remain motionless to minimise discomfort (Masarani and Dinneen, 2007). Colic occurs as a consequence of obstruction in the passageway of the urinary tract by calculi (Masarani and Dinneen, 2007). In patients with severe colic, nausea and vomiting might also occur (El-Barky et al., 2014). It has been found that approximately 90% of patients presenting with colic also present with gross or microscopic haematuria (Masarani and Dinneen, 2007), while urine infection causing shivers, sweating and fever was observed in patients to a lesser extent (Steggall and Omara, 2008).

A routine assessment consisting of an exhaustive history and physical examination is usually performed on patients presenting with symptoms related to urolithiasis. Preliminary diagnosis is confirmed with proper radiological imaging methods. This allows for the physician to rapidly and definitively diagnose the patient's calculus/calculi and ensures substantial

knowledge, including size, location, composition and renal anatomy, to decide the most suitable treatment modalities (Mehmet and Ender, 2015).

There is a wide array of imaging methods that are currently used in the diagnosis of calculus formation. Previously, abdominal plain films of the kidneys, ureters and bladder and intravenous urography (IVU) were the most effective radiological techniques for diagnosis of ureteral stones. The sensitivity of a plain radiograph of the kidney, ureter and bladder in evaluation of acute flank pain ranges from 45 – 60% (Mutgi et al., 1991). Calculi can be classified based on images on radiograms, where calcium calculi are radiopaque, and directly visible, and non-calcium calculi, including uric acid and xanthine stone, are radiolucent, and not directly visible. The disadvantages of abdominal plain films are that radiolucent stones (which make up approximately 10% of stones) cannot be visualised. Additionally, abdominal and pelvic calcifications and overlaying bowel gas or stool can make it difficult to identify a ureteral calculus effectively (Masarani and Dinneen, 2007). Furthermore, it has been shown that in some cases, image quality in obese patients is impaired and there has been difficulty in differentiating pelvic vascular calcifications from stones in the proximal ureter close to the kidneys (Mandeville et al., 2011). Despite the disadvantages, a plain film may be adequate to determine size, shape and location of urinary calculi in certain patients (Masarani and Dinneen, 2007) (Figure 1.3).

Intravenous urography provides information on site, degree and nature of obstruction and has a detection rate of 70 – 90% (Figure 1.4), which is significantly higher than with plain films (Miller et al., 1998). However, it has been closely associated with undesirable characteristics including radiation exposure, risk of nephrotoxicity, contrast reaction and time period of the procedure. Also, similar to plain films, only radiopaque calculi are visible (Masarani and Dinneen, 2007). The incidence of contrast-induced renal failure is reported to be approximately 1% (Levy et al., 1996) while in

patients with pre-existing renal failure and diabetes mellitus, the risk of nephrotoxicity is 25% (Barrett and Carlisle, 1993). The incidence of contrast reactions with IVU is 5 – 10%, and these include vomiting, urticaria, bronchospasm and anaphylaxis (Shehadi and Toniolo, 1980).



Figure 1.3 *Kidney, ureter and bladder x ray of a patient presented with left loin pain showing 7mm radiopaque stone located lateral to the tip of transverse process of L2 (Masarani and Dinneen, 2007).*

With the rapid development in technologies such as ultrasound and non-contrast computed tomography (NCCT), a much wider range of imaging studies are now available to physicians (Zilberman et al., 2011). The main

advantage ultrasound has over other imaging techniques is that there is no radiation exposure, and therefore, it is commonly utilised for stone diagnosis in pregnant women and also in follow up procedures to ensure signs of ureteral obstruction have resolved after treatment (Macneil and Bariol, 2011). Ultrasound can be used to detect calculi located in the pelvis, calices, and specific locations of the ureter including the pelviureteric junction (where ureter leaves the kidney) and vesicoureteric junction (where ureter enter the bladder) (Sheafor et al., 2000, Ray et al., 2010).



Figure 1.4 Intravenous urography film showing left nephrogram and contrast coming down the ureters to the level of the stone, after administration of intravenous contrast medium (Miller et al., 1998).

IVU was previously the standard imaging modality for assessing stones, however, the advancement in NCCT recently has given this technique better specificity and sensitivity for stone detection (Shine, 2008) (Figure 1.5). In comparison to IVU, NCCT not only has a higher sensitivity and specificity for detection of calculi, but also does not require intravenous contrast medium administration. Furthermore, NCCT has a shorter examination time and the ability to determine radiolucent stones, in addition to being able to define density and internal formation of the stone and the distance of stone from the skin (Masarani and Dinneen, 2007). The reliability of NCCT in detection of calculi is undeniable with sensitivity, specificity and positive predictive values being 96%, 100% and 100% respectively (Worster et al., 2002). Nonetheless, the most distinct advantage of NCCT is that it does not require administration of intravenous contrast medium and therefore, does not induce nephrotoxicity and contrast reactions (Masarani and Dinneen, 2007).



Figure 1.5 *Non-contrast computer tomography of a patient showing left ureteral calculus (Miller, 2003).*

The most important limitation of NCCT is the lack of ability to assess degree of obstruction and functional evaluation of the kidney which could possibly compromise clinical management (Masarani and Dinneen, 2007). Another major drawback is the higher radiation exposure compared to other methods. Nevertheless, newer protocols requiring less radiation exposure without affecting efficacy are rapidly developing. These procedures are termed low-dose and ultra low-dose NCCT respectively and been shown to reduce radiation exposure by 50% and 95% compared to standard-dose NCCT, and this is without influencing calculi detection rates (Meagher et al., 2001, Kluner et al., 2006, Poletti et al., 2007). Other disadvantages of NCCT include its questionable 24-hour availability due to the demand for a radiologist to accurately interpret films and its significantly higher cost. However, it has been suggested that its advantages in terms of reduced time of diagnosis and manpower are enough to lower indirect costs when compared to other methods (Pfister et al., 2003).

Current Treatments

Choosing treatment modalities for urolithiasis is dependent upon stone size, location and composition, along with the patient's symptoms and comorbidities. In addition to that, the degree of ureteral obstruction and ureteral colic presented in specific patients also contribute to the choice of treatment (Steggall, 2002). In recent years, there has been a great improvement in the technology for minimally invasive treatment modalities utilised in the management of urinary stones.

Intervention treatments involved in treating larger urinary stones include shock wave lithotripsy (SWL), ureteroscopy, open nephrolithotomy, and percutaneous nephrolithotomy. The SWL procedure involves the use of shock waves to fragment urinary stones (Figure 1.6). Ureteroscopy is performed with an endoscope passing through the urethra and bladder

and into the ureter for removal of the stone (Macneil and Bariol, 2011). Open nephrolithotomy is a surgical procedure, where the kidney is dissected to remove the stone, whereas in percutaneous nephrolithotomy the stone is removed by a small puncture wound through the skin. The nephrolithotomy procedures are more invasive in nature due to the requirement of skin penetration (Macneil and Bariol, 2011).

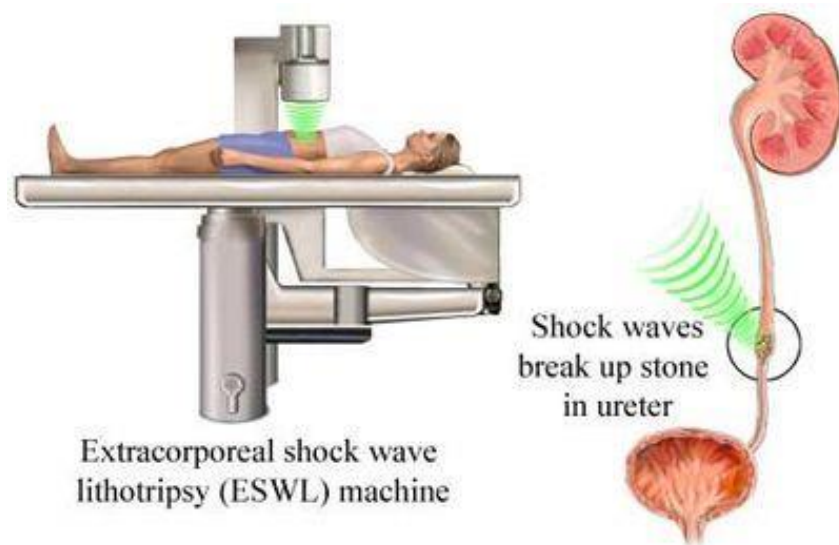


Figure 1.6 Extracorporeal shock wave lithotripsy procedure. Shock waves are utilised to crush stones in both the kidney and ureter. In both circumstances, stone fragments are required to pass through the ureter in the urine to the bladder for expulsion (Aboumarzouk et al., 2012).

As shown in Figure 1.7, the less invasive methods, including ureteroscopy, pyeloscopy and shock wave lithotripsy (SWL), are more popular compared to the invasive treatments including open and percutaneous nephrolithotomy (Lee and Bariol, 2011). Since the introduction of SWL as a treatment for managing stone disease, it has been the most common first line treatment for patients (Preminger et al., 2007). Although SWL is

the least invasive method, its efficacy is highly dependent on stone burden, location, composition and the efficacy of different lithotripters. It is predominantly dependent on the size of stone, where the stone-free rate is excellent in stones of less than 1cm (Preminger et al., 2007). Stone clearance rates for larger stones are much better with ureteroscopy (Parker et al., 2004, Kijvikai et al., 2007, Salem, 2009, Aboumarzouk et al., 2012). SWL is the least invasive treatment method, but nevertheless has been associated with complications including instances of tissue injury, bleeding, urinary tract obstruction, adjacent organ injury and urinary tract infections. However, it is notable that the increased risk of these complications is associated with comorbidities including hypertension, diabetes mellitus, obesity and coronary heart disease. Although these acute effects of SWL are known, it is established that treatment with this modality does not affect renal functions in the long term (Mehmet and Ender, 2015). There are contradicting results as to whether complication rates are higher in ureteroscopy than in SWL or if they are similar (Aboumarzouk et al., 2012, Parker et al., 2004).

In the introductory years of ureteroscopy, the total cost for the procedure was higher than SWL (Bierkens et al., 1998). However, the evolvement of new model flexible ureteroscopies, laser fibers and baskets in the last decade has been tremendous and its total cost is currently less than the total charges for shock wave lithotripsy (Salem, 2009, Lotan et al., 2002). Physicians are inclined to recommend ureteroscopy for stones larger than 2cm. It is considered an effective alternative for percutaneous nephrolithotomy in patients with significant comorbidities including anticoagulation, cardiopulmonary disease, advanced age, obesity and renal malformations (Palmero et al., 2012, Akman et al., 2012).

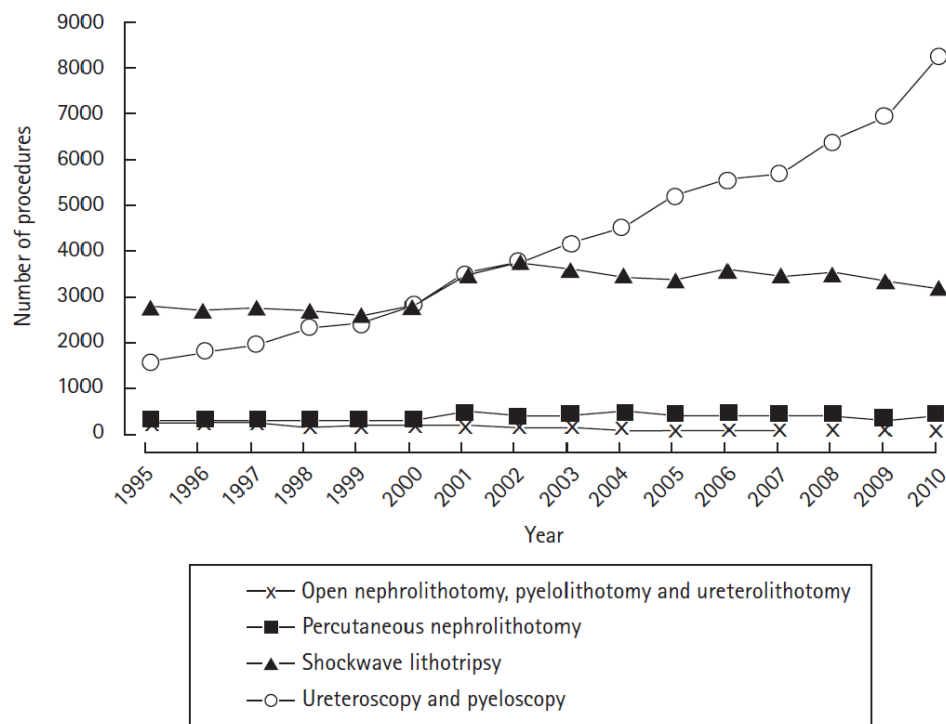


Figure 1.7 Number of procedures claimed under Medicare Australia for different stone treatment modalities between 1995 and 2010 (Lee and Bariol, 2011).

Medical Expulsive Therapy

The likelihood of spontaneous calculus passage within 4 weeks ranges between 50% - 95% and this has been shown to be highly dependent on stone size, where stones that are less than 3mm in diameter are expected to pass spontaneously while stones greater than 6mm generally do not (Simon et al., 1997, Miller and Kane, 1999). Thus, in cases of asymptomatic and smaller stones, 'watchful' waiting might be recommended and might be extended by addition of medical expulsive therapy to improve passing and expulsion of stones (Canda et al., 2007).

The primary purposes of medical expulsive therapy are to increase the rate of stone expulsion along the ureter to avoid ureteral obstruction and

reduce ureteral colic, the excruciating pain in the ureter frequently accompanying stone expulsion (Michel and de la Rosette, 2006). Furthermore, effective medical expulsive therapy is also expected to benefit patients, as it will reduce the need for hospitalization and surgical procedures. While the pathophysiology of this disease is still not fully understood, several drugs are currently used clinically in patients with ureteric stones. These act by relaxation of the ureter and augmentation of the hydrostatic physical force proximal to the calculus (Sivula and Lehtonen, 1967). Currently, the two most common drug classes used in medical expulsive therapy are calcium channel blockers and α_1 -adrenoceptor antagonists. Both these drug classes are thought to act by inducing smooth muscle relaxation in the ureters to allow stone passage.

The most commonly used α_1 -adrenoceptor antagonist is tamsulosin, and results from various studies suggest that tamsulosin displays a significant efficacy in stone expulsion rate and colic episodes in comparison to untreated patients (Hollingsworth et al., 2006, Michel and de la Rosette, 2006, Parsons et al., 2007, Seitz et al., 2009, Al-Ansari et al., 2010, Kaneko et al., 2010). While these studies indicate the efficacy of tamsulosin, other investigations suggested that tamsulosin does not increase the rate of stone expulsion over placebo (Hermanns et al., 2009, Wang et al., 2009). Documented side effects due to administration of tamsulosin include dizziness, nausea and vomiting, headache and transient hypotension, with the latter being the most commonly reported (Hollingsworth et al., 2006, Singh et al., 2007, Seitz et al., 2009). The incidence of side effects by tamsulosin in urolithiasis patients is approximately 4% (Hollingsworth et al., 2006, Singh et al., 2007, Seitz et al., 2009).

While the use of tamsulosin in medical expulsive therapy is the most studied α_1 -adrenoceptor antagonist, a randomised control trial demonstrated equal efficacy between tamsulosin, terazosin and doxazosin

in comparison to the trial groups (Yilmaz et al., 2005). The use of silodosin, a selective α_{1A} -adrenoceptor antagonist, as a substitute for tamsulosin has also received increasing attention and has been reported to significantly increase calculus expulsion rate (Gupta et al., 2013, Dell'Atti, 2015). While no severe complications with silodosin administration were recorded, it was associated with a significantly higher incidence of retrograde ejaculation as an adverse effect (Gupta et al., 2013, Dell'Atti, 2015).

The only calcium channel blocker studied in association with urolithiasis is nifedipine, which has demonstrated some benefit in calculus expulsion and relieving colic (Dellabella et al., 2005, Porpiglia et al., 2006, Osorio et al., 2008, Seitz et al., 2009). However, multiple studies have reported superior efficacy of tamsulosin in comparison to nifedipine in calculus passage facilitation (Saita et al., 2004, Dellabella et al., 2005, Ye et al., 2011). Furthermore, the occurrence of adverse effects including nausea and vomiting, asthenia, dyspepsia, headache, drowsiness and euphoria, appears to be higher with administration of nifedipine (15.2%) than tamsulosin (4%) (Singh et al., 2007). Hence, the focus of medical expulsive therapy is predominantly on α_1 -adrenoceptor antagonists, despite promising results of calcium channel blockers.

The majority of the randomized studies performed on α_1 -adrenoceptor antagonists and calcium channel blockers were not blinded and had unclear allocation concealment, which leads to the probability of an overestimation of treatment efficacy and limits the strength of final conclusions regarding their benefits. Additionally, these studies were predominantly small and based on single clinical centres. Interestingly, a recent study which took into account these limitations reported that administration of neither tamsulosin nor nifedipine affected the number of patients requiring further intervention for clear calculus passage after 4 weeks, suggesting similar rates of spontaneous calculus passage between

trial groups (tamsulosin, nifedipine, placebo) (Pickard et al., 2015). Consequently, the current guidelines for medical expulsive therapy by both European (EAU) and American (AUA) Urological Associations outline the role of tamsulosin as the most viable option in clinical settings. Medical expulsive therapy is not as commonly administered in Australia in comparison to the United States and in European countries due to its inefficacy. The most common pharmacological treatment prescribed to patients with urolithiasis are pain killers. However, there have been reports where tamsulosin (Floxamatra) was prescribed to patients with smaller stones in Australia.

In addition to these agents, non-steroidal anti-inflammatory drugs (NSAIDs) are often used for relief of pain from inflammation and therefore also sometimes administered to patients with ureteric stones with accompanying colic (Holmlund and Sjodin, 1978). NSAIDs are thought to relieve pain by inhibiting the formation of inflammatory mediators. Diclofenac is the most commonly used NSAID in ureteral colic and has been shown to be efficient in reducing pain with no or very few side effects in multiple clinical trials (Basar et al., 1991, Vignoni et al., 1983, Sommer et al., 1989, Walden et al., 1993). However, NSAIDs are useful only in relieving pain and do not alter stone expulsion rate in ureteral colic patients (Laerum et al., 1995, Hermanns et al., 2009). Additionally, most studies only involved a short-term prescription of NSAIDs to avoid many of the adverse effects associated with prolonged treatment (Bos and Kapoor, 2014). Therefore, although many studies have shown that NSAIDs in combination with either α_1 -antagonists or calcium channel blockers can be effective (Porpiglia et al., 2006, Yilmaz et al., 2005, Hwang et al., 2012), their efficacy in reducing calculus expulsion time as a monotherapy is still yet to be manifested.

A novel candidate in medical expulsive therapy is PDE5 inhibitors, which can cause smooth muscle relaxation in the ureter (Gratzke et al., 2007).

However, the potential value of this class of drugs, particularly in clinical settings, is still in its infancy. In one study, it was reported that there is no significant difference in calculus expulsion rate between patients administered with only tamsulosin and patients who were treated with combined tamsulosin and the PDE5 inhibitor tadalafil (Kumar et al., 2014). Further clinical investigations are required to assess the efficacy of PDE5 inhibitors in medical expulsive therapy. Another novel medical expulsive therapy drug that has emerged in clinical trials is β_3 -adrenoceptor antagonist, mirabegron (Tabner et al., 2016) but similar to PDE5 inhibitors, its efficacy has not been thoroughly investigated.

In summary, the efficacy of currently used pharmacological agents, namely tamsulosin, nifedipine and NSAIDs, in increasing stone expulsion rate, is still questionable. Additionally, while their adverse effects are considered mild, there are still significant factors to be considered in the choice of treatment as they can affect patients' quality of life. Based on current findings, it is evident that medical expulsive therapy is most advantageous for patients with smaller calculi. Additionally, it has been shown that administration of both tamsulosin and nifedipine can benefit patients who undergo the shock wave lithotripsy procedure, by facilitating the passage of fragmented stones along the ureter (Kupeli et al., 2004, Micali et al., 2007). Therefore, the development of a more effective medical expulsive therapy agent, that can both increase stone expulsion rate and relieve colic. will be advantageous for patients with smaller ureteral stones by avoiding more invasive procedures. In order to achieve this aim, a greater understanding of the mechanisms involved in the contractility of the ureter is required.

1.2 Structure and Function of the Ureter

Ureteral Structure

The right and left ureters are attached to the renal pelvis of the right and left kidneys and together, these organs, the ureters and kidneys, compose the upper urinary tract. The main, and possibly only, function of the ureter is the unidirectional transport of urine from the kidneys to the bladder (Santicioli and Maggi, 1998). The mammalian ureter consists of two main cell types: a multilayered water-tight transitional epithelium called the urothelium, which is surrounded by subepithelial connective tissues and smooth muscle cells (Woolf and Davies, 2013). There are two distinct layers of smooth muscle cells: an inner longitudinal layer and an outer circular layer, surrounded by fibrous tissues, as shown in Figure 1.8. The longitudinal smooth muscle is responsible for ureteral shortening and plays a role in movement of urine, while circular smooth muscle that coats the ureteral walls contracts to generate pressure (Vargiu et al., 2015).

Pyeloureteral Motility

Ureteral peristalsis is the propagation of smooth muscle contraction and relaxation waves down the ureteric muscular tube to propel urine from the kidneys to the bladder. The basic process of ureteral peristalsis has long been thought to be myogenic in nature, where it is initiated by renal pacemaker cells and conducted to the ureters. This mechanism requires both the kidneys and the ureters and is therefore known as the pyeloureteral complex (Santicioli and Maggi, 1998, Lang et al., 1998).

Spontaneous ureteral contractility originates at the renal pelvis, due to electrical activity at the pacemaker sites. Electrical activity is propagated in

a cell-to-cell manner distally to the otherwise inactive smooth muscle cells in the ureter, creating a contractile wave and the ability for urine propulsion to the bladder (McHale et al., 2006). Although the pathophysiology of renal colic is not completely understood, it is proposed that increased luminal pressure and volume in the ureter that occur due to the obstruction can induce an increase in peristaltic wave amplitude and frequency (Rose and Gillenwater, 1973). This can significantly amplify pain, and therefore cause ureteral colic (Rose and Gillenwater, 1973).

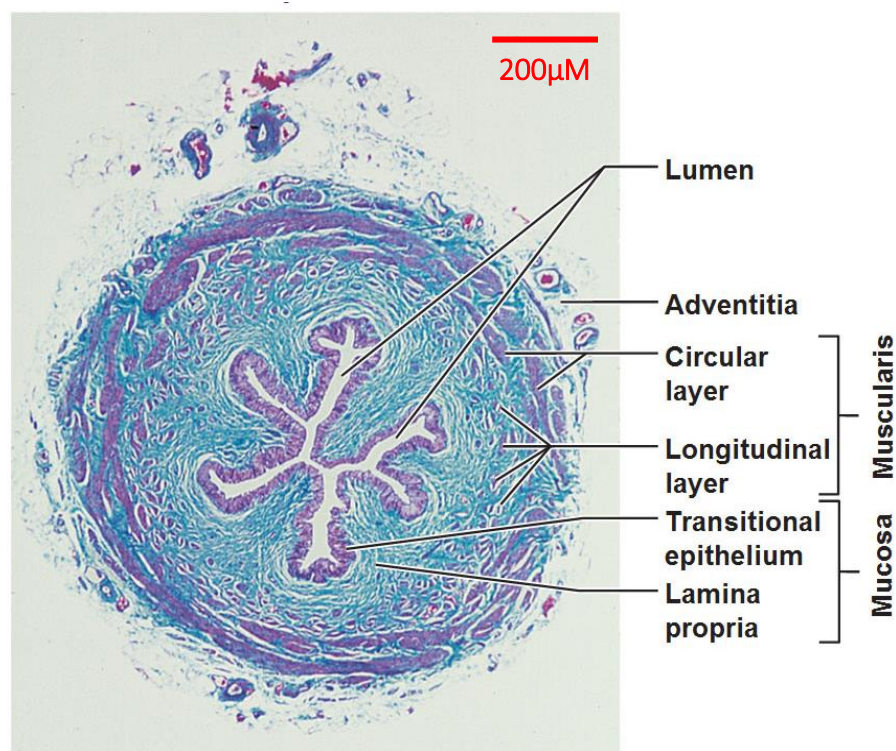


Figure 1.8 Microscopic image of transverse section of the human ureter. Smooth muscle tissues are surrounded by fibrous tissues and lined with a layer of transitional epithelium.

According to current literature, the most likely candidate for the renal pacemaker cells of the pyeloureteral complex are 'atypical' smooth muscle cells, which are not to be confused with the 'typical' smooth muscle cells

that compose the ureter (Lang and Klemm, 2005). The pacemaker sites are located at the border where the calyces attach to the base of the papilla, called the pelvi-calyceal junction. This region is very weakly contractile with few 'typical' smooth muscle cells (McHale et al., 2006). These 'atypical' smooth muscles were identified by neurobiotin injection and it was observed that they have similar morphological and electrical characteristics to cardiac sino-atrial cells, which are responsible for cardiac excitation (Berridge, 2008). This results in a loose resemblance between the regulation of cardiac and pyeloureteral excitation. Electrical and mechanical activities are initiated by these spontaneously active cells in the pelvi-calyceal region of the renal pelvis and conducted to distal regions in the ureter that are not normally active unless driven by pacemakers (Berridge, 2008). Therefore, it is only when the electrical impulse is conducted to the ureter that there is an initiation of contractions that propagates along the ureter to the uretero-vesical junction as a peristaltic wave (McHale et al., 2006).

As shown in the microelectrode recordings by Klemm and colleagues in Figure 1.9, the 'atypical' smooth muscle cells, which account for approximately 10% of cells in the pyeloureteral complex, display spontaneous activity characterized by slow rising and repolarizing phases called 'pacemaker' potentials. On the contrary, the action potentials in the ureters are 'driven', consisting of a rapidly rising initial spike and followed by a period of membrane oscillations and a plateau phase (Klemm et al., 1999). The pacemaker potentials have been shown to be resistant to cholinergic, noradrenergic and neuronal blockers, suggesting a purely myogenic origin. Additionally, it has been shown that peristalsis occurs normally in isolated pyeloureteral complex, further indicating its myogenic origin (Santicioli and Maggi, 1998).

Under conditions of normal diuresis, a conduction block appears to exist between the renal pelvis and the ureter. Every pacemaker contraction of

the renal pelvis does not necessarily propagate to the ureter (Santicioli and Maggi, 1998). A urine flow-dependent mechanism triggers ureteral peristalsis, where stretching forces exerted by increasing volume of urine on the pyeloureteral region increase the coupling strength to enable an incoming pacemaker wave to pass to the ureter (Constantinou and Yamaguchi, 1981).

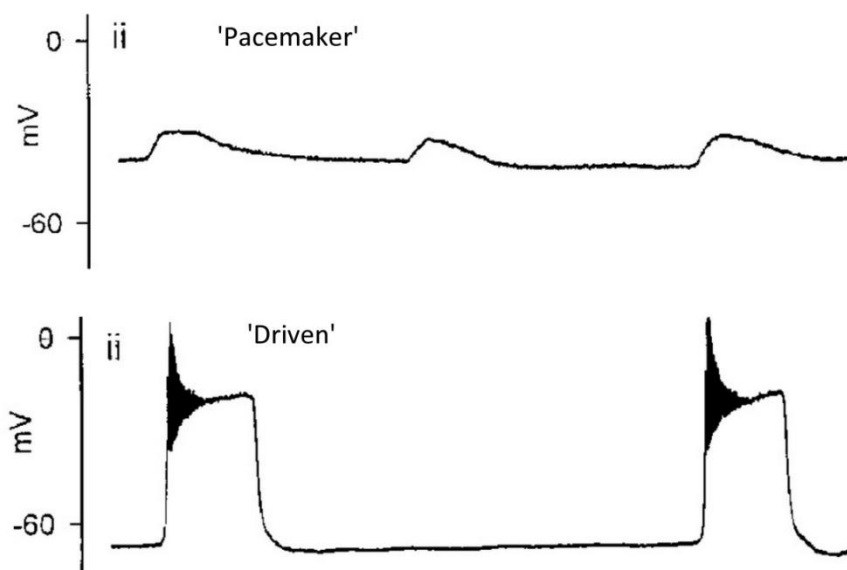


Figure 1.9 Typical intracellular microelectrode recordings of 'pacemaker' potentials in the renal pelvis and 'driven' action potentials in the ureter (Klemm et al., 1999).

In the past decade, it was discovered that there is a third population of electrically-active cells which play a fundamental role in pyeloureteric autorhythmicity. These cells display numerous similar morphological features to the interstitial cells of Cajal (ICC) present in the intestinal tract and thereby are called ICC-like cells. ICC-like cells have been observed in the lamina propria of the guinea pig renal pelvis and pelvi-calyceal junction, but not identified in the ureter (Klemm et al., 1999). While both

'atypical' smooth muscle cells and ICC-like cells display spontaneous electrical activity, 'atypical' smooth muscle cells show high frequency oscillations of the membrane potential, while the ICC-like cells show low frequency oscillations. Therefore, it was suggested that in the absence of 'atypical' smooth muscle cells, ICC-like cells provide a compensatory pacemaker mechanism to maintain peristaltic waves in 'typical' SMCs, but are not normally involved in setting the pace (Lang and Klemm, 2005). It was also proposed that these ICC-like cells could be responsible for conducting and amplifying pacemaker signals to initiate action potentials in the 'typical' smooth muscle cells of the ureter (McHale et al., 2006).

The 'typical' smooth muscle cells in the ureter propagate proximal to distal peristaltic waves from the pacemaker cells in the pelvi-calyceal junction, pumping urine from the renal pelvis to the bladder for storage and micturition (Woolf and Davies, 2013). Smooth muscle cells play an integral part in most mammalian organ systems, including the ureters. Clinical disorders including hypertension, bronchial asthma and preterm birth are results of altered smooth muscle contractility by a change in balance between contractile and relaxing agents, and so it is highly likely that this mechanism is involved, to some extent, in lodgement of ureteric stones (Lang and Klemm, 2005).

1.3 Innervation of the Ureter

Until the last couple of decades, neurogenic control of smooth muscle cell contraction in the ureter has remained elusive. While it is known that the myogenic properties of the pyeloureteral complex are sufficient for normal ureteral peristalsis, stimulation of sensory nerves by neurotransmitters released in the presence of stone or infections can excite latent pacemakers present in the smooth muscle, giving rise to antiperistaltic waves or urine backflow (Santicioli and Maggi, 1998). Additionally, the primarily inactive distal regions of the ureter have also been shown to be dependent on neurogenic mechanisms for the modulation of contractility (Santicioli and Maggi, 1998).

The mammalian ureter is innervated mainly by unmyelinated fibers originating from the renal, ovarian or spermatic and sympathetic plexuses (Wharton et al., 1981). It has also been suggested that the lower part of the ureter in cat and guinea pigs may receive a pelvic innervation (Wharton et al., 1981). In humans, the distal ureter receives a greater density of innervation compared to the proximal ureter, suggesting the possibility of a major contribution from the pelvic plexus (Edyvane et al., 1992). The ureters have both an efferent and afferent innervation including adrenergic, cholinergic and non-adrenergic non-cholinergic components (Gray, 1918).

Adrenergic Innervation

Adrenoceptors are responsible for mediating responses to endogenous catecholamines, namely noradrenaline and adrenaline. The two types of adrenoceptors are α -adrenoceptor and β -adrenoceptor. α -adrenoceptors were initially thought to be involved mostly in excitatory functions including vasoconstriction, uterine and urethral smooth muscle contraction and pupil

dilation. A group of α -adrenoceptors responsible for inhibitory responses were discovered and these were termed α_2 -adrenoceptors, while the excitatory receptors were labelled α_1 -adrenoceptors. β -adrenoceptors, on the other hand, mediate inhibitory functions including vasodilation, uterine and urethral smooth muscle relaxation and bronchodilation but are also involved in excitatory effects in the cardiac system (Civantos Calzada and Aleixandre de Artinano, 2001).

There are currently four known α_1 -adrenoceptor subtypes: α_{1A} -, α_{1B} -, α_{1D} -, and α_{1L} -adrenoceptors, the latter representing a functional phenotype of the α_{1A} -adrenoceptor (Civantos Calzada and Aleixandre de Artinano, 2001). α_1 -adrenoceptors are coupled predominantly by G-proteins of the $G_{q/11}$ family to activate the phospholipase C mechanism (Docherty, 2010) whose effect on smooth muscle contraction will be discussed in detail later. α_1 -adrenoceptor antagonists are most well-known for their use in benign prostatic hyperplasia patients as this receptor type is widely distributed in the prostatic smooth muscle. The blockade of these receptors also reduces urethral constriction and enhances urine flow (Pinheiro and Martins Pisco, 2012).

The net result of noradrenaline application on the isolated human ureter is generally contraction, indicating the functional dominance of α_1 -adrenoceptors over β -adrenoceptors (Hernandez et al., 1992). Molecular characterisation and immunohistochemical staining for α_1 -adrenoceptors in the human ureter showed that α_{1D} and α_{1A} -adrenoceptor subtypes were prevalent over the α_{1B} -adrenoceptor subtype (Figure 1.10) (Sigala et al., 2005, Itoh et al., 2007, Park et al., 2007). Radioligand binding studies indicated a heterogenous distribution of all three subtypes with the greatest density of α_1 -adrenoceptors at the distal ureter in comparison to the proximal and mid ureters (Sigala et al., 2005).

Although the expression of α_{1D} -adrenoceptors is observed to be the greatest of the subtypes, a functional study performed on isolated human ureter suggested that the α_{1A} -adrenoceptor is the subtype that plays the major functional role in mediating contractions (Sasaki et al., 2011). In this study, it was found that the α_{1A} -selective antagonist silodosin had a greater affinity than the α_{1D} -selective antagonist, BMY-7378 and the non-selective α_1 -adrenoceptor antagonist, prazosin (Sasaki et al., 2011). These results support the efficacy of the current drug used in medical expulsive therapy, tamsulosin, which is an α_{1A} -adrenoceptor selective antagonist, in increasing the expulsion rate of ureteral stones in patients (Dellabella et al., 2003, Autorino et al., 2005, Yilmaz et al., 2005, Agrawal et al., 2009).

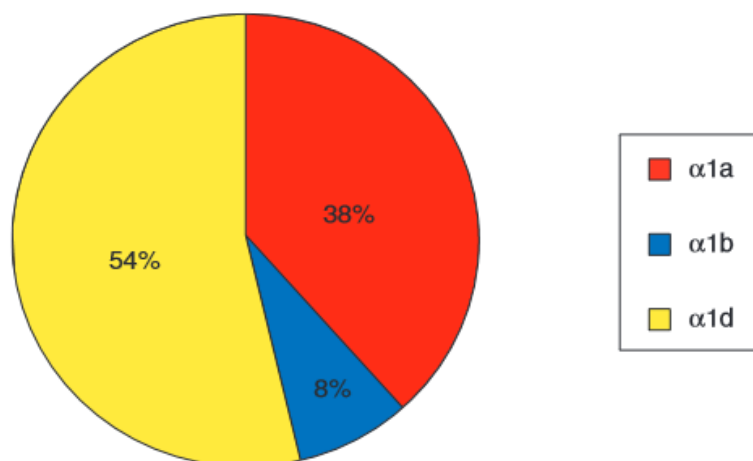


Figure 1.10 α_1 -adrenoceptor subtypes mRNA density in the human ureter. α_{1D} -adrenoceptor has the highest mRNA expression followed by α_{1A} -adrenoceptor then α_{1B} -adrenoceptor (Itoh et al., 2007).

α_2 -adrenoceptors are involved in various physiological functions, particularly in the cardiovascular system and central nervous system. They are linked to G_i -proteins which inhibit adenylyl cyclase, causing a decrease in cAMP concentration upon activation and thereby, results in smooth

muscle contraction (Gyires et al., 2009). Additionally, they are found on the sympathetic nerve terminals where they inhibit noradrenaline release. This acts as a negative feedback mechanism for noradrenaline release (Gyires et al., 2009). There are currently three recognised α_2 -adrenoceptor subtypes and these are α_{2A} -, α_{2B} -, and α_{2C} -adrenoceptors. α_{2A} -adrenoceptors are thought to be responsible for most classical effects of α_2 -adrenoceptors including antihypertensive effects, bradycardia, antinociception and also regulation of the gastrointestinal tract (Gyires et al., 2009). On the other hand, the activation of α_{2B} - and α_{2C} -adrenoceptors are likely to modify the α_{2A} -adrenoceptor-mediated effect by either contributing or negating these reactions (Gyires et al., 2009).

There is limited data on α_2 -adrenoceptors in the ureter but a study performed on porcine ureteric tissues suggested that there could be a possible role for these receptors in the maintenance of ureteral wall tone (Macneil and Bariol, 2011). Rauwolscine, the α_2 -adrenoceptor selective antagonist, blocked tone of the ureter without affecting the phasic activity induced upon a submaximal dose of noradrenaline addition. However, in this same study, B-HT920, B-HT933 and clonidine, all α_2 -adrenoceptor agonists failed to elicit contractions, even in the presence of low potassium and phenylephrine concentrations to induce initial depolarisation (Hernandez et al., 1992). Thus, the effect of raulwolscine may have been due to a non-selective action on α_1 -adrenoceptors.

There are three well-known β -adrenoceptor subtypes: β_1 -, β_2 - and β_3 -adrenoceptor (Michel, 2011). The stimulation of β -adrenoceptors induces an increase in cAMP via adenylate cyclase and therefore, causes smooth muscle relaxation (Michel, 2011). β_1 -adrenoceptors are mainly involved in cardiac excitation as they are present in cardiac pacemakers and the myocardium. β_2 - and β_3 -adrenoceptors have both been observed to induce relaxation in the detrusor muscle of the urinary bladder (Michel, 2011).

The two outcomes of β -adrenoceptor agonist application on the ureter are pacemaker activity alteration and smooth muscle relaxation (Michel, 2011). Functional studies involving β -adrenoceptors are mostly organ bath experiments involving isolated ureteric smooth muscle tissues, hence alteration of pacemaker activity by β -adrenoceptor stimulation has not been well characterised. Although there is generally a functional dominance of α_1 -adrenoceptors in the ureter, it has been suggested that there is a stronger component of β -adrenoceptor expression in the distal ureter (Michel, 2011). MRNA for all three β -adrenoceptor subtypes are expressed in the human ureter and the existence of β_2 -adrenoceptors has been clearly demonstrated by receptor-binding assay (Park et al., 2000). While there are no radio-ligand binding data on β_3 -adrenoceptors, the co-existence of this subtype with β_2 -adrenoceptors has been well characterised through pharmacological functional experiments.

A study performed on porcine isolated ureteral smooth muscle cells showed that CGP-12177A and CL-316243, β_2 - and β_3 -selective agonists, produced a greater relaxation effect on potassium chloride-induced contractions in comparison to dobutamine, a β_1 -adrenoceptor selective agonist (Wanajo et al., 2004). In addition to that, isoprenaline-induced relaxations were antagonized by the β_2 -selective antagonist ICI-118,551 and the β_3 -selective antagonist SR 58894A but not by the β_1 -adrenoceptor antagonist CGP20712A (Wanajo et al., 2004). Furthermore, it was demonstrated in a different study that the β_2/β_3 -adrenoceptor agonist KUL-7211, was more potent and has a more selective effect on the pig ureter *in vitro* and *in vivo* in comparison to the non-selective β -agonists isoprenaline and CL-316243 or the β_2 -adrenoceptor selective agonist terbutaline (Wanajo et al., 2011). KUL-7211 also potently and selectively relaxed isolated human ureteric tissues (Tomiyaama et al., 2003a). In a study on human ureter, the relaxant effects of procaterol, a β_2 -adrenoceptor selective agonist, and TRK-380, a β_3 -selective agonist were greater in comparison to β_1 -selective agonists (Matsumoto et al., 2013). These

results suggest that the subtypes mediating relaxation in pig and human ureter are β_2 - and β_3 -adrenoceptors.

Cholinergic Innervation

There are five currently known subtypes of muscarinic receptors that have been cloned and defined pharmacologically: M_1 , M_2 , M_3 , M_4 and M_5 . Muscarinic receptor subtypes M_1 , M_3 and M_5 receptors are coupled to $G_{q/11}$ guanine nucleotide binding proteins and cause contraction through phospholipase C activation in smooth muscle cells (Uchiyama and Chess-Williams, 2004). M_2 and M_4 receptor subtypes, on the other hand, are coupled to G_i proteins to attenuate smooth muscle relaxation responses through inhibition of adenylate cyclase (Uchiyama and Chess-Williams, 2004). In the majority of smooth muscle studies, M_3 receptors have been shown to mediate contraction (Eglen et al., 1994).

Immunocytochemical analysis of human ureters detected the presence of all five known subtypes of muscarinic receptors, whereas reverse transcription polymerase chain reaction analysis was only able to detect mRNA for M_2 , M_3 and M_5 subtypes (Sakamoto et al., 2006). In this same study, it was found that M_5 receptor expression was greater than other subtypes (Sakamoto et al., 2006). Functional studies performed on the isolated pig ureter showed that the M_1 receptor mediates the tonic response while phasic activity could involve M_1 , M_2 , M_3 or M_4 (Hernandez et al., 1993). Therefore, the functional role of muscarinic receptors in the ureter is still not clearly understood, especially in the human ureter.

5-Hydroxytryptamine

The major source of 5-HT in human is found in the gastrointestinal tract (Barnes, 2001). There are currently six known classes of G protein-

coupled 5-HT receptors present in mammals, namely 5-HT₁, 5-HT₂, 5-HT₄, 5-HT₅, 5-HT₆ and 5-HT₇. There is an amazing diversity of signalling mechanisms for each 5-HT receptor class as they are each divided into different subtypes (Roth, 1994). The general signalling mechanisms of each 5-HT class are shown in the table below (Table 1.1).

Table 1.1 5-HT receptor classes and their general signalling mechanisms (Roth, 1994).	
Receptor Class	Signalling Mechanism/s
5-HT₁	Inhibits adenylate cyclase
5-HT₂	Activates phospholipase C
5-HT₃	Ligand-gated ion channel
5-HT₄	Activates adenylate cyclase Activates protein kinase A
5-HT₅	Inhibits adenylate cyclase
5-HT₆	Activates adenylate cyclase
5-HT₇	Activates adenylate cyclase

In isolated strips of human ureter, 5-HT induced concentration dependent contractions (Barnes, 2001, Gidener et al., 1999). The 5-HT induced effect was antagonised by the 5-HT₂ antagonist, ketanserin and the mixed 5-HT₁/5-HT₂ receptor antagonist methysergide, but was unaltered by the blockage of 5-HT₃ and 5-HT₄ receptors (Gidener et al., 1995).

Intravenous and topical administration of the 5-HT_{2A} antagonist ritanserine resulted in an inhibition of contractions in the pig isolated intravesical ureter (Hernandez et al., 2003). In addition, the 5-HT_{2A} agonist DOI,

increased the frequency of ureteral contractions in pig ureter *in vivo* in a dose-dependent manner (Hauser et al., 2002). These outcomes suggest a possible role of the 5-HT_{2A} receptor in mediating contractility of porcine ureter, although further studies are required to clearly determine the receptor type responsible for these 5-HT-induced contractile responses.

Nitric Oxide

There is ample evidence that nitric oxide (NO) is a major inhibitory neurotransmitter in many tissues of the urogenital tract and acts via an increase in cGMP (Andersson and Arner, 2004). Neuronal NO synthase (NOS)-positive neuronal axons have been detected in human and porcine isolated ureters and *in vitro* findings suggest a possible role for NO in ureteral relaxation (Stief et al., 1996, Iselin et al., 1996). NOS-reactive nerves were also detected via immunohistochemistry in the both human and pig distal and intravesical ureter and NO was suspected to have a possible role regulating the valve of the ureterovesical junction, where the ureter enters the bladder (Hernandez et al., 1995, Iselin et al., 1997, Iselin et al., 1998). Besides its effect on the ureterovesical junction, *in vitro* studies on mice and dog have also suggested that NO production is decreased in ureteral obstruction, and have shown its importance in reducing intra-ureteral pressure (Chang et al., 2002, Felsen et al., 2003, Xingyu et al., 2012).

Histamine

Histamine is an inflammatory mediator released by basophils and mast cells. The two histamine receptor subtypes that have been found in smooth muscle cells are the H₁ and H₂ histamine receptors. The H₁ receptor has been shown to stimulate bronchial smooth muscle

contraction and causes bronchoconstriction while stimulation of H₂ receptors is primarily involved in vasodilation (Marquardt, 1983).

Electron microscopy of human ureters has demonstrated that urine can penetrate subepithelially in ureteral damage as observed with ureteral stones, and induce mast cell degranulation with the release of histamine (Ugaily-Thulesius and Thulesius, 1988, Ugaily-Thulesius et al., 1988). This process is likely one of the mechanisms of renal colic. A functional study showed that in human ureteral smooth muscle, the H₁-receptor was predominant, as contraction induced by histamine stimulation completely masks the relaxation effect of H₂-receptors (Bertaccini et al., 1983). In animal models, H₁-receptor stimulation caused excitation in dog and sheep ureter, while H₂ receptor stimulation initiated relaxation in dog and buffalo ureters (Dodel et al., 1996, Benedito et al., 1991, Phadnis et al., 1995). The association between histamine and inflammation suggests that histamine receptors could play a significant role in ureteral colic in patients with ureteral stones.

Prostaglandins

Prostaglandins are lipid compounds derived from fatty acids which have various roles including regulation of contraction and relaxation of smooth muscle. The formation of prostaglandins requires cyclooxygenase (COX) enzymes. The COX enzyme converts arachidonic acid to an intermediate PGH₂ which is metabolised to PGD₂, PGE₂, PGF_{2α} and PGI₂ (Matsuoka and Narumiya, 2007). The seven prostaglandin G-protein coupled receptors and their corresponding prostaglandin types are: DP receptors (PGD), EP₁, EP₂, EP₃ and EP₄ receptors (PGE), FP receptors (PGF) and IP receptors (PGI) (Matsuoka and Narumiya, 2007). There are two isoforms of the COX enzyme: COX-1, a constitutive form expressed in many tissue and organs, and COX-2, an inducible form induced by various stimuli including stretching of muscle, mucosal injuries and inflammatory

mediators, and nerve stimulation (Norregaard et al., 2006, Nakada et al., 2002).

A study performed on isolated human ureteric tissues showed that administration of PGE₂ and PGF_{2α} increased phasic and tonic activity (Cole et al., 1988). However, another study showed that PGE₁ and PGE₂ caused inhibition of contractile activity in normal human ureter *in vitro*, while PGF_{1α} and PGF_{2α} caused increased contraction (Abrams and Feneley, 1975). Additionally, studies have shown that PGE₂ increased contractility in ureteral obstruction and relaxed normal and non-obstructed ureters in pigs through the subtype EP₃ receptor (Lowry et al., 2005, Ankem et al., 2005).

The drug class NSAIDs, that is currently used for pain relief in ureteral colic, act by inhibiting the COX enzyme. Indomethacin, a non-selective COX inhibitor has been shown to dose-dependently inhibit ureteral contractility in isolated human and sheep ureters (Angelo-Khattar et al., 1985, Al-Ugaily et al., 1986). Diclofenac, also a COX inhibitor, was shown to cause relaxation in human ureters (Sivrikaya et al., 2003). COX-2 enzyme expression is up-regulated in human and rat ureter following ureteral obstruction, a complication that could accompany ureteric stones (Norregaard et al., 2006, Nakada et al., 2002). The increase of this isoform has led to investigations as to whether COX-2 selective inhibitors will be effective, with fewer side effects, in managing ureteral colic. NS-398 and celecoxib, COX-2 selective inhibitors, have been shown to be equipotent with non-selective inhibitors indomethacin and diclofenac, at reducing agonist-induced contractions and inhibiting prostaglandin release in pig and human ureters (Mastrangelo et al., 2000, Nakada et al., 2000, Jerde et al., 2005, Lee et al., 2010). These analyses indicate the importance of prostaglandins in regulation of ureteral smooth muscle contraction although clinical studies have been focused on the ability of COX inhibitors to relieve pain, and not in increasing stone expulsion rate.

Neuropeptides

The role of neuropeptides in the ureter have not been thoroughly investigated. However, it has been reported that the release of calcitonin gene-related peptide (CGRP) inhibits ureteral motility in the guinea pig ureter in vitro and in vivo (Hua and Lundberg, 1986, Maggi et al., 1994). However, CGRP-positive nerve density was reported to be significantly lower in the human ureter compared to other species and these were detected mainly around blood vessel and submucosa and not at the smooth muscle (Edyvane et al., 1992, Tainio et al., 1992).

1.4 Intracellular Signalling Pathways in Smooth Muscle Contraction

Many organs in the body possess layers of smooth muscle cells and smooth muscle contraction is vital for most bodily functions and abnormalities can contribute to a wide range of diseases. The change in intracellular Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$ is currently thought to be the principal foundation that initiates contraction and relaxation in smooth muscle cells of the ureter (Somlyo and Somlyo, 1994). Figure 1.11 depicts a brief overview of intracellular mechanisms controlling general smooth muscle contraction and the different, but not necessarily independent, intracellular pathways thought to be involved in mediation of smooth muscle contraction are discussed below.

Phospholipase C Pathway

Agonists that act on G-protein coupled receptors (GPCR) can induce both Ca^{2+} sensitization and activation of phospholipase C (PLC) via the $\text{G}_{q/11}$ family of GPCR to induce contraction (Somlyo and Somlyo, 1994). Receptors that are suggested to activate the PLC pathway in the ureter are α_1 -adrenoceptor, muscarinic receptor subtypes M_1 , M_3 and M_5 , 5-HT₂ receptor, histamine H_1 receptor and prostaglandin receptors.

PLC activation catalyses the breakdown of phosphatidylinositol-4,5-bisphosphate (PIP_2) into intracellular second messengers inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces Ca^{2+} release from the sarcoplasmic reticulum, increasing $[\text{Ca}^{2+}]_i$ available for binding calmodulin. The association of calcium-calmodulin complex with the catalytic subunit of myosin light chain kinase (MLCK) activates this kinase to phosphorylate the myosin light chain (MLC_{20}) (Matsumura and

Hartshorne, 2008). Phosphorylated MLC₂₀ activates myosin to form cross-bridges with actin filaments (Sweeney et al., 1994). Myosin utilizes adenosine triphosphate (ATP) hydrolysis as the chemical energy source to facilitate its attachment and interaction with actin filaments. The formation of these cross-bridges is the mediator for motor responses in smooth muscle cells (de Godoy and Rattan, 2011).

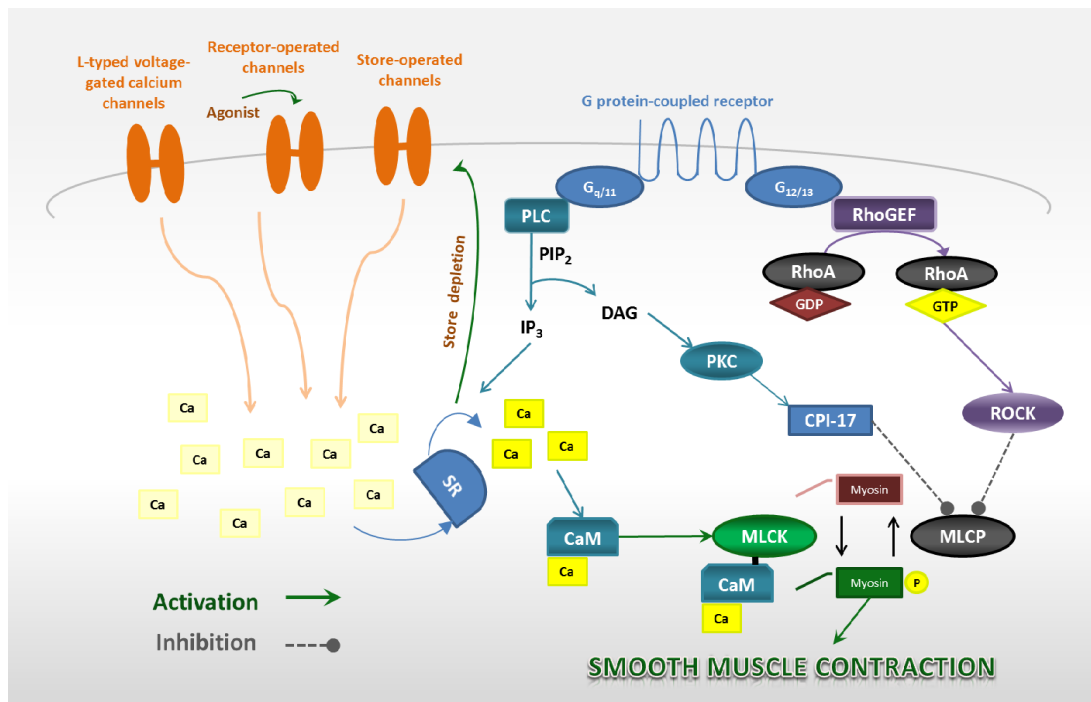


Figure 1.11 Intracellular mechanisms involved in smooth muscle contraction. CaM, calmodulin; DAG, diacylglycerol; GPCR, G protein-coupled receptor; IP₃, inositol triphosphate; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; PIP₂, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; RhoGEF, Rho-specific guanine nucleotide exchange factor; ROCK, Rho-associated protein kinase; SR, sarcoplasmic reticulum

The other product of PIP₂ breakdown, DAG, activates protein kinase C (PKC). PKC is a constitutively active protein kinase that phosphorylates CPI-17, a potent inhibitor of myosin light chain phosphatase (MLCP). The activated form of MLCP in the smooth muscle binds phosphorylated MLC₂₀ at its myosin-binding site and dephosphorylates it to terminate smooth muscle contraction (MacDonald et al., 2001). Therefore, the final consequence of DAG is enhancement of contractions at constant [Ca²⁺]_i. It has been found that Ca²⁺ release can be dissociated from Ca²⁺ sensitization hence, although IP₃ induces Ca²⁺ release, it does not necessarily initiate Ca²⁺ sensitization which is an important process to maintain smooth muscle contraction (Kobayashi et al., 1991).

Role of Calcium Channels

Smooth muscle contraction is highly dependent on the release of Ca²⁺ from the sarcoplasmic reticulum to the cell cytoplasm. This depletes the intracellular Ca²⁺ store and replenishment of Ca²⁺ from the extracellular space is required (McFadzean and Gibson, 2002). The best characterised Ca²⁺ entry pathway is via the L-type calcium channel which is a voltage-dependent channel. This channel is sensitive to depolarization of the smooth muscle cells, allowing Ca²⁺ entry (McFadzean and Gibson, 2002). However, non-voltage-gated channels permeable to Ca²⁺ are also present in smooth muscle cells including receptor-operated channels (ROCs) and store-operated channels (SOCs).

ROCs are proposed to have the properties of non-selective cation currents, with varying degrees of Ca²⁺ selectivity (McFadzean and Gibson, 2002). ROCs can be activated by agonists that act on GPCR (McFadzean and Gibson, 2002). Multiple extensive studies have been performed in various smooth muscle cell types and have suggested promising ROC activators. These include noradrenaline, acetylcholine and histamine acting via α_1 -adrenoceptors, M₂ muscarinic and H₁ receptors respectively

(Wang and Large, 1991, Komori et al., 1992, Zholos and Bolton, 1997). Although IP₃-mediated release of Ca²⁺ from the sarcoplasmic reticulum is not necessary for activation of ROC, the increase in [Ca²⁺]_i has been observed to augment the channel current, and hence, calcium entry (Pacaud and Bolton, 1991).

Intracellular Ca²⁺ store depletion stimulates Ca²⁺ influx across the plasma membrane to maintain [Ca²⁺]_i during prolonged agonist application and also aids refilling of stores upon agonist withdrawal (Putney et al., 2001). SOC_s are currently thought to be the pathway of this calcium entry from the extracellular space into the smooth muscle, occurring via a family of non-selective cation channels. Reduced intracellular stores of calcium, regardless of the cause, activate SOC to allow Ca²⁺ current across the cell membrane (McFadzean and Gibson, 2002). It was suggested that the single spanning membrane protein, STIM1, acts as the Ca²⁺ sensor to trigger SOC activation (Minke, 2006).

Rho/Rho-kinase/Calcium Sensitization Pathway

RhoA is a monomeric G-protein that belongs to the family of GTPases that include RhoA, RhoB and RhoC isoforms. It is the best understood isoform due to its significant roles in smooth muscle motility (de Godoy and Rattan, 2011). RhoA acts like a molecular switch, changing between the active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)-bound state (Gidener et al., 1995). The activation of RhoA protein is through the Rho-specific guanine nucleotide exchange factor (RhoGEF) which is known to catalyse the switch between GDP and GTP on RhoA. Activated GTP-bound RhoA interacts with multiple effectors including Rho-associated protein kinase (ROCK), protein kinase N, rhotekin, rhophilin, citron and phospholipase D (Wettschureck and Offermanns, 2002).

ROCK is one of the most investigated RhoA effectors and exists in two isoforms; ROCK1 and ROCK2, with both present in smooth muscle (Yoshii et al., 1999). ROCK, when activated by RhoA, inactivates MLCP by phosphorylation of the myosin binding subunit. ROCK has also been shown to directly phosphorylate MLC₂₀ to induce actin-myosin cross-bridging which is the motor response for smooth muscle contraction (Amano et al., 1996, Somlyo and Somlyo, 2000). Hence, ROCK induces Ca²⁺ sensitization and enhances smooth muscle contraction independent of [Ca²⁺]_i. Although the second messenger DAG also plays a similar role via PKC as a regulator of MLCP activity, it only plays a minor role in comparison to ROCK, as shown in the rabbit trachea (Iizuka et al., 1999). There have been suggestions of the possibility of cross-talk between the PKC and Rho/Rho-kinase pathways. The attenuation of PKC-induced contraction in the internal anal sphincter was observed not only in the presence of a PKC inhibitor but also by a ROCK inhibitor (Patel and Rattan, 2007).

There are several levels where regulation of the Rho/Rho-kinase pathway can occur. Figure 1.9 shows a brief overview of RhoA regulation. At the GPCR, the activating agonist or antagonist will activate or inhibit the pathway respectively. As mentioned previously, RhoGEF is responsible for the activation of Rho by exchanging GDP with GTP on the protein. RhoGEF activity is controlled by protein kinases, phosphatidylinositol kinases and dimerization (Zheng, 2001). The next level of regulation is at the RhoA protein. It is well established that G-proteins, including G₁₂-family, G₁₂ and G₁₃ which seem to be ubiquitously expressed, couple to GPCRs and activate Rho (Buhl et al., 1995, Fromm et al., 1997). Though not fully understood, there is increasing evidence that Rho can be activated by G_q and the G₁₁ families too (Katoh et al., 1998, Mao et al., 1998). One of the negative regulatory mechanisms of Rho activity is via GTPase activating proteins (GAPs). GAPs inactivate the GTP-bound Rho by increasing RhoA intrinsic GTPase activity (Olofsson, 1999). GTPase-

dissociation inhibitor (GDI) inhibits GAP-stimulated GTP hydrolysis and is therefore a positive regulator for RhoA (Olofsson, 1999).

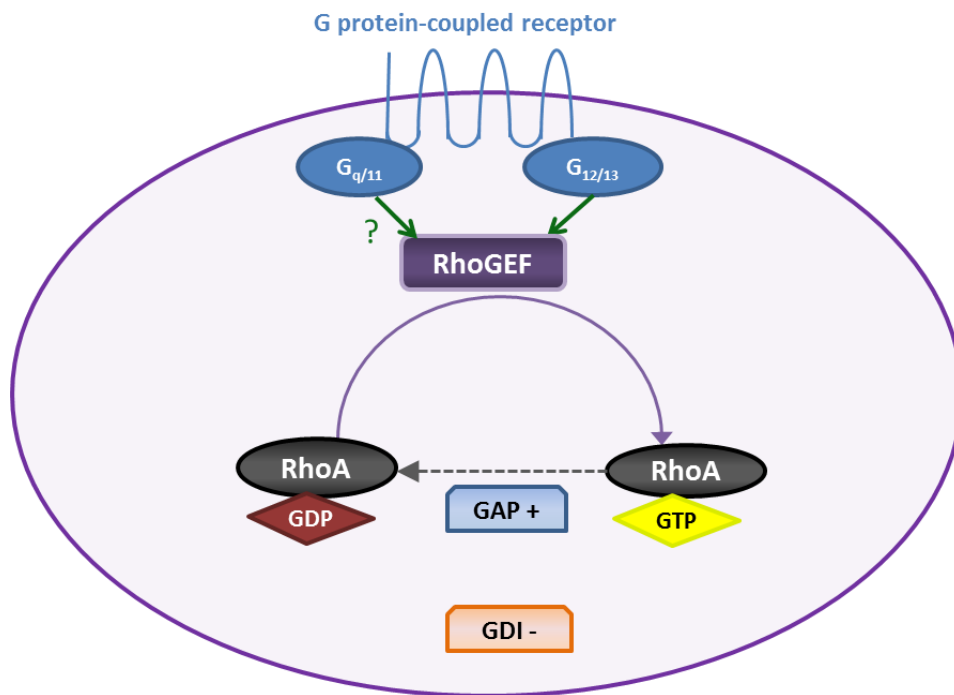


Figure 1.12 Regulation of RhoA protein activity. Various G-protein receptor classes activate RhoA through Rho-specific guanine nucleotide exchange factor (RhoGEF). GTPase activating protein (GAP) and GTPase-dissociation inhibitor (GDI) are negative and positive regulators respectively.

The ROCK pathway has been shown to be upregulated in pathophysiological conditions involving bladder smooth muscle dysfunction and in response to ureteral outlet obstruction (Zhang and DiSanto, 2011). Immunohistochemistry and Western blotting experiments in ureteric tissues have indicated the presence of both ROCK isoforms, ROCK-1 and ROCK-2, in human and sheep (Ferguson et al., 1998, Levent

and Buyukafsar, 2004). The presence of ROCK in the ureter suggests that it could have a significant functional role in smooth muscle contraction. The role of ROCK in ureteric obstruction was recently shown, where Y-27632, a specific ROCK inhibitor, suppressed contraction in obstructed rabbit ureter and a greater ROCK expression was also seen via Western blotting (Turna et al., 2007). Y-27632 was also shown to attenuate both spontaneous and electrical field stimulated contractile responses in the human ureter (Zhang and DiSanto, 2011). However, no studies have linked the role of Rho/Rho-kinase pathway to the stimulation of α_1 -adrenoceptors, muscarinic, histamine, prostaglandin and 5-HT receptors in the ureter.

Cyclic Nucleotides

Smooth muscle relaxation occurs either as a consequence of the removal of a contractile stimulus or by a direct action of a substance that stimulates inhibitory mechanisms (Webb, 2003). Nevertheless, the relaxation process requires a decrease in $[Ca^{2+}]_i$ and increase in MLCP activity. The two main intracellular second messengers involved in smooth muscle relaxation are the cyclic nucleotides, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) (Carvajal et al., 2000).

As shown in Figure 1.13, the conversion of ATP to cAMP is catalysed by adenylate cyclase. Adenylate cyclase is activated by the α subunit of the G_s protein. cAMP generated by adenylate cyclase activate cAMP-dependent protein kinase A (PKA) (Sassone-Corsi, 2012). PKA causes a decrease in $[Ca^{2+}]_i$ and thereby induces smooth muscle cell relaxation through a number of mechanisms. PKA activates Ca^{2+} -activated K^+ (K_{Ca}) channels where the efflux of K^+ is related to the concentration of Ca^{2+} . Upon activation, these channels act as a negative feedback mechanism, hyperpolarizing the cell membrane and inhibiting Ca^{2+} entry through voltage-gated channels and thereby inactivating the PLC pathway, which

causes inhibition of smooth muscle contraction. Additionally, PKA also decreases the activity of RhoA and hence, the Rho/Rho-kinase pathway which stimulates smooth muscle contraction (Sanborn et al., 2005).

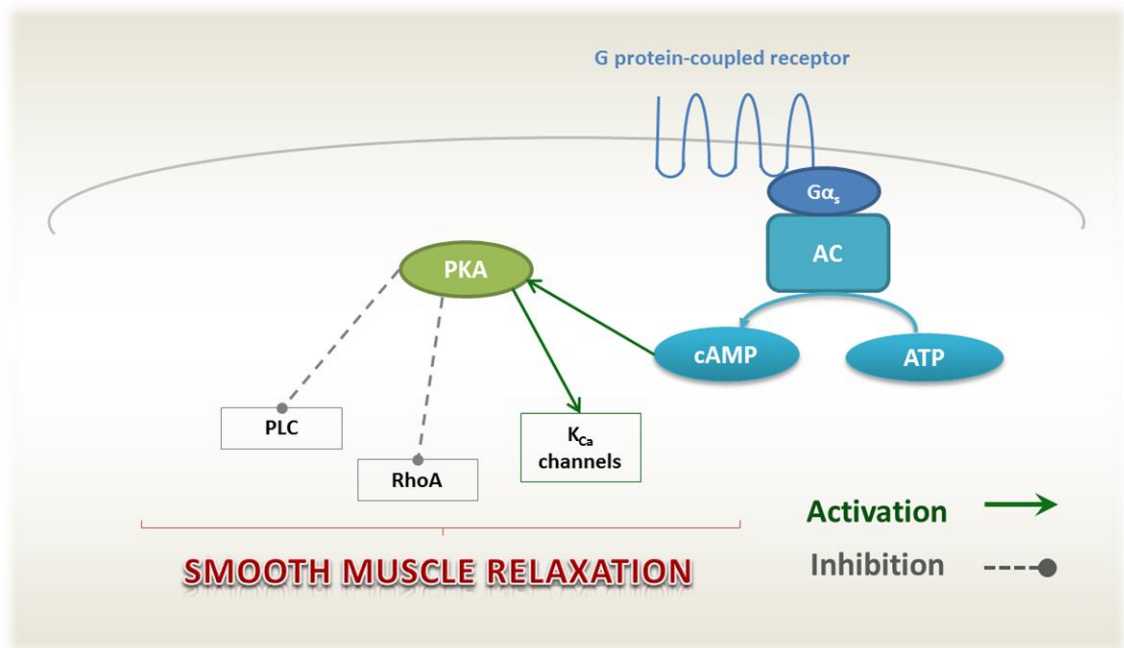


Figure 1.13 Cyclic adenosine monophosphate pathway. GPCR stimulation causes activation of adenylyl cyclase by $G\alpha_s$ subunit. Adenylyl cyclase converts adenosine triphosphate to cAMP which stimulates protein kinase A (PKA) to activate and inhibit mechanisms that induce SMC relaxation.

The cyclic guanosine monophosphate (cGMP) relaxation pathway is summarised in Figure 1.14. cGMP is generated by the activity of guanylate cyclases. Nitric oxide is responsible for the activation of soluble guanylate cyclase (sGC) which catalyses the conversion of guanosine triphosphate (GTP) to cGMP. cGMP triggers the relaxation of SMC via the activation of

protein kinase G (PKG). Through the activation and inhibition of several proteins, PKG decreases $[Ca^{2+}]_i$ (Carvajal et al., 2000).

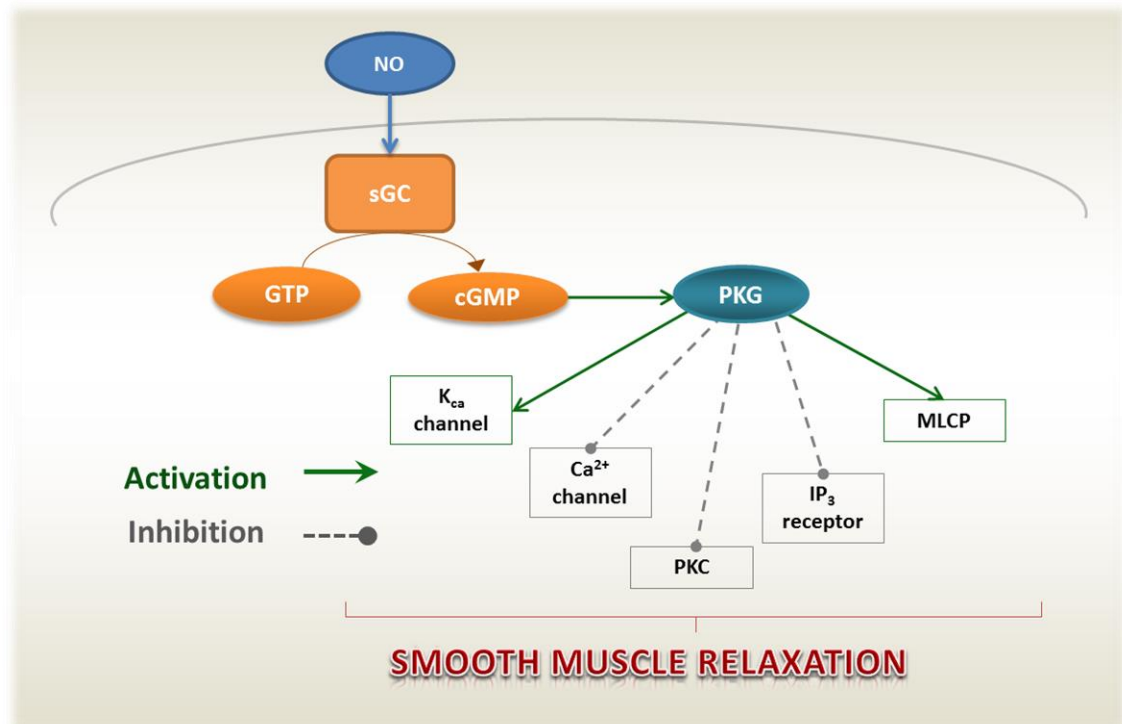


Figure 1.14 Cyclic guanosine monophosphate pathway. Nitric oxide (NO) stimulates soluble guanylate cyclase (sGC) which converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). cGMP then activates protein kinase G (PKG) which triggers a reduction in $[Ca^{2+}]_i$ via several mechanisms.

Firstly, PKG activates K_{Ca} channels where K^+ entry causes hyperpolarization and inhibition of Ca^{2+} entry through voltage-gated channels (Kannan and Johnson, 1995). Besides that PKG also inhibits membrane Ca^{2+} channel activity preventing the entry of Ca^{2+} (Horowitz et al., 1996). PKG also inhibits PKC and blocks IP_3 receptors which have been discussed previously as important pathways for contraction of

smooth muscle cells. It may also activate MLCP which is responsible for dephosphorylating the MLC_{20} , causing relaxation of smooth muscle cells (Carvajal et al., 2000). The relaxing effect of K_{ATP} channels was also reported in the isolated human ureter in vitro and also in intact ureters of anesthetized pigs in vivo (Weiss et al., 2002) which suggest that K_{ATP} channels have a role in the intracellular mechanism of smooth muscle contraction in the ureter. In addition to this, it is suggested that hydrogen disulphide is a potent inhibitory neurotransmitter in the pig intravesical ureter via the activation of K_{ATP} channels (Fernandes et al., 2014).

1.5 Summary

Ureteral colic is a common symptom presented clinically in patients affected by ureteral calculi which causes significant discomfort. The ureter is perhaps the least researched organ of the urinary tract. Although numerous molecular and functional studies have been performed on receptors at the membrane level of ureteric smooth muscle cells, there are many gaps in the knowledge of the control of ureteric smooth muscle contractile activity, and particularly, in the intracellular signal transduction modulating these responses. It is important to have a greater understanding of the intracellular mechanisms controlling ureteral smooth muscle contraction, as this may reveal novel mechanisms and identify targets for development of more effective pharmacological agents for expulsion of ureteral calculi and the treatment of ureteral colic.

1.6 Research Aims

The overall aim of this study was to elucidate the mechanisms controlling ureteral motility by filling the gaps of current research, with a view to identify novel targets for the development of suitable drugs for medical expulsive treatment of ureteral stones.

Specifically, isolated pig ureteral tissues were utilised in this study with the aims to:

- 1) Optimise *in vitro* methodology for use of the pig model in studies of ureteral contractility (regions of ureter, tissue orientation, age groups).
- 2) Functionally characterise agonist-mediated responses of the ureter, particularly those mediated via adrenoceptors, muscarinic, histamine, and 5-HT receptors.
- 3) Functionally and molecularly characterise the intracellular signalling pathways linked to these receptors, in particular the Rho-kinase pathway / calcium sensitisation.
- 4) Functionally investigate the effect of the urothelium and adenosine triphosphate (ATP) on agonist-mediated contractile activity.

CHAPTER 2:

Methods Optimization

2.1 General Methods

Tissue Preparation

Female pig urinary bladders, with ureters and urethra attached, were obtained from the local abattoir (Highchester Meats, Beau-Dessert, Queensland). These were immediately placed in ice-cold Krebs bicarbonate solution (4°C) and transported on ice to Bond University for experimental procedures. Both right and left ureters were detached from the bladders and peri-ureteric fat was removed with care to avoid disruption of the smooth muscle layer of the ureter. The ureters were divided into proximal and distal segments, where proximal ureter was determined as the first 5cm of tube leaving the kidneys, and distal ureter was determined as the 5cm just before entering the bladder. These were dissected into 4mm long tissue strip sections. The tissue strips were mounted in 8ml organ baths (EZ-baths, Global Towns, CA) containing Krebs-bicarbonate solution, which was maintained at 37°C and continuously gassed with 95% O₂ and 5% CO₂ (Figure 2.1).



Figure 2.1 Set-up of four 8ml organ baths (EZ-baths, Global Towns, CA) with isometric transducers attached.

The ends of each tissue strip were attached with suture to an isometric transducer and tissue holder. A resting tension of 1 - 1.5g was set on the tissues and they were allowed to equilibrate for 45 minutes before the experimental protocol was commenced. During this resting period, tissues were washed with fresh Krebs solution every 15 minutes. Isometric tension generated by the tissues was measured using the isometric transducers connected to a PC and recorded using Powerlab LabChart® 6 software (AD Instruments). At the end of all experiments, wet tissue weights were measured and recorded after removing moisture by dabbing with tissue paper.

Buffers and Drugs

Krebs-bicarbonate solution was used for all experiments. The composition is shown in the following table (Table 2.1).

<i>Table 2.1 Composition of Krebs bicarbonate solution</i>	
Compound	Molarity
Sodium chloride	118.4mM
D(+)Glucose	11.7mM
Sodium bicarbonate	25.0mM
Potassium chloride	4.6mM
Magnesium sulphate	2.4mM
Potassium dihydrogen phosphate	1.2mM
Calcium chloride	1.9mM

The chemicals and drugs used in the experiments are listed in the table below (Table 2.2). Stock solutions were made up in distilled water (dH₂O) where possible, and if not, in dimethyl sulfoxide (DMSO) or ethanol. Further dilutions were made in distilled dH₂O. Where stock solutions were made up with DMSO or ethanol, vehicle controls were incorporated into the experimental design.

<i>Table 2.2 List of Drugs and Chemicals (continued on following page)</i>			
Compound	Molecular Weight	Supplier	Catalogue Number
Sodium chloride	58.44	Sigma-Aldrich	S9888
D-(+) Glucose	180.16	Sigma-Aldrich	G8270
Sodium bicarbonate	84.01	Sigma-Aldrich	S5761
Potassium chloride	74.55	Sigma-Aldrich	P9541
Magnesium sulphate	120.37	Sigma-Aldrich	M7506
Potassium dihydrogen phosphate	136.09	Sigma-Aldrich	P9791
Calcium chloride solution (1M)	110.98	Sigma-Aldrich	21115
Phenylephrine hydrochloride	203.67	Sigma-Aldrich	P6126
Carbamoylcholine chloride	182.65	Sigma-Aldrich	C2409
5-hydroxytryptamine hydrochloride	212.68	Abcam	153-98-0
Histamine dihydrochloride	184.07	Sigma-Aldrich	H7250
Indomethacin	357.79	Sigma-Aldrich	I7378
N-Nitro-L-arginine	219.20	Sigma-Aldrich	N5501

Table 2.2 List of Drugs and Chemicals (continued from previous page)

Compound	Molecular Weight	Supplier	Catalogue Number
Methiothepin mesylate salt	452.65	Sigma-Aldrich	M149
Ketanserin (+)-tartrate salt	545.51	Sigma-Aldrich	S006
RS-102221	649.08	Tocris	1050
Ondansetron hydrochloride dihydrate	365.85	Sigma-Aldrich	O3639
GR-113808	393.50	Tocris	1322
SB 699551	584.66	Sigma-Aldrich	SML0853
SB-399885 hydrochloride	482.81	Sigma-Aldrich	SML0604
SB-269970 hydrochloride	388.95	Sigma-Aldrich	S7389
α-methyl-5-hydroxytryptamine maleate	306.32	Tocris	0557
Y-27632 dihydrochloride	320.26	Tocris	1254
Fasudil hydrochloride	327.83	Tocris	0541
Adenosine 5'-triphosphate disodium salt hydrate	551.14	Sigma-Aldrich	A2383
Apyrase from potatoes	3.0-40.0 Units/mg	Sigma-Aldrich	A6132
Suramin sodium salt	1429.17	Sigma-Aldrich	S2671
1,3-Dimethyl-8-phneylxanthine (8-phenyltheophylline)	256.26	Sigma-Aldrich	P2278
Dimethyl sulfoxide ACS reagent	78.13	Sigma-Aldrich	472301

Data Analysis

In preliminary experiments, in response to phenylephrine and other agonists, isolated ureteral strips from pigs develop spontaneous phasic contractions (Figure 2.2). Figure 2.2 showing the raw trace of contractile response to phenylephrine is the typical trace seen in response to all agonists including 5-HT and carbachol. The appearances of the phasic activities in response to all agonists are similar. The increase in amplitude of contractions in this spontaneous contractility did not necessarily correlate with increasing concentrations of agonist. Therefore, contractile activities of these tissues were expressed as area under the curve (AUC), by weight in grams seconds (g s) and frequency in Hertz (Hz), the number of phasic contractions in a second (see Appendix 1 for calculation of AUC using LabChart software). For all experiments within this thesis, the n number of preparations stated represents the number of different animals that were used.

Appropriate statistical analyses for each set of data were performed and these will be discussed in detail in their respective chapters. In this methods optimization chapter, paired or unpaired (Chapter 2.5) Student's *t*-tests were performed to identify statistically significant differences, where $p < 0.05$ was deemed a significant difference.

2.2 Optimization of Responses of Ureteral Tissue

When subjected to phenylephrine and other agonists (listed in later sections), tissue strips from the porcine ureter produced bursts of spontaneous contractile activity with increasing frequency of contraction that was dependent on increasing concentrations of the agonist. However, the amplitude of phasic contraction was independent of agonist concentration, and there was no consistent pattern of increase or decrease of amplitude at various concentrations of phenylephrine. Basal tension was not significantly increased with increased agonist concentrations. While amplitude appears to increase with concentration in Figure 2.2, this was not necessarily concentration-dependent in the majority of experiments. Additionally, within one concentration of agonist, the amplitude of the phasic contractions varied. This was apparent for all agonists that induced any contractile response.

In preliminary investigations of cumulative concentration-response curves, it was notable that the tissues were desensitised at pre-maximal concentrations of phenylephrine following cumulative addition of agonist (Figure 2.2 and 2.4). Therefore, in order to produce accurate and consistent results, responses to single concentrations of agonist were performed in all subsequent experiments with porcine ureters, where each concentration of agonist was added for 5 minutes and washed for 10 minutes before the following concentration was added. This allowed for accurate measurement of contractile activity to each agonist concentration, as a proper baseline was exhibited before each burst of phasic contractions (Figure 2.3).

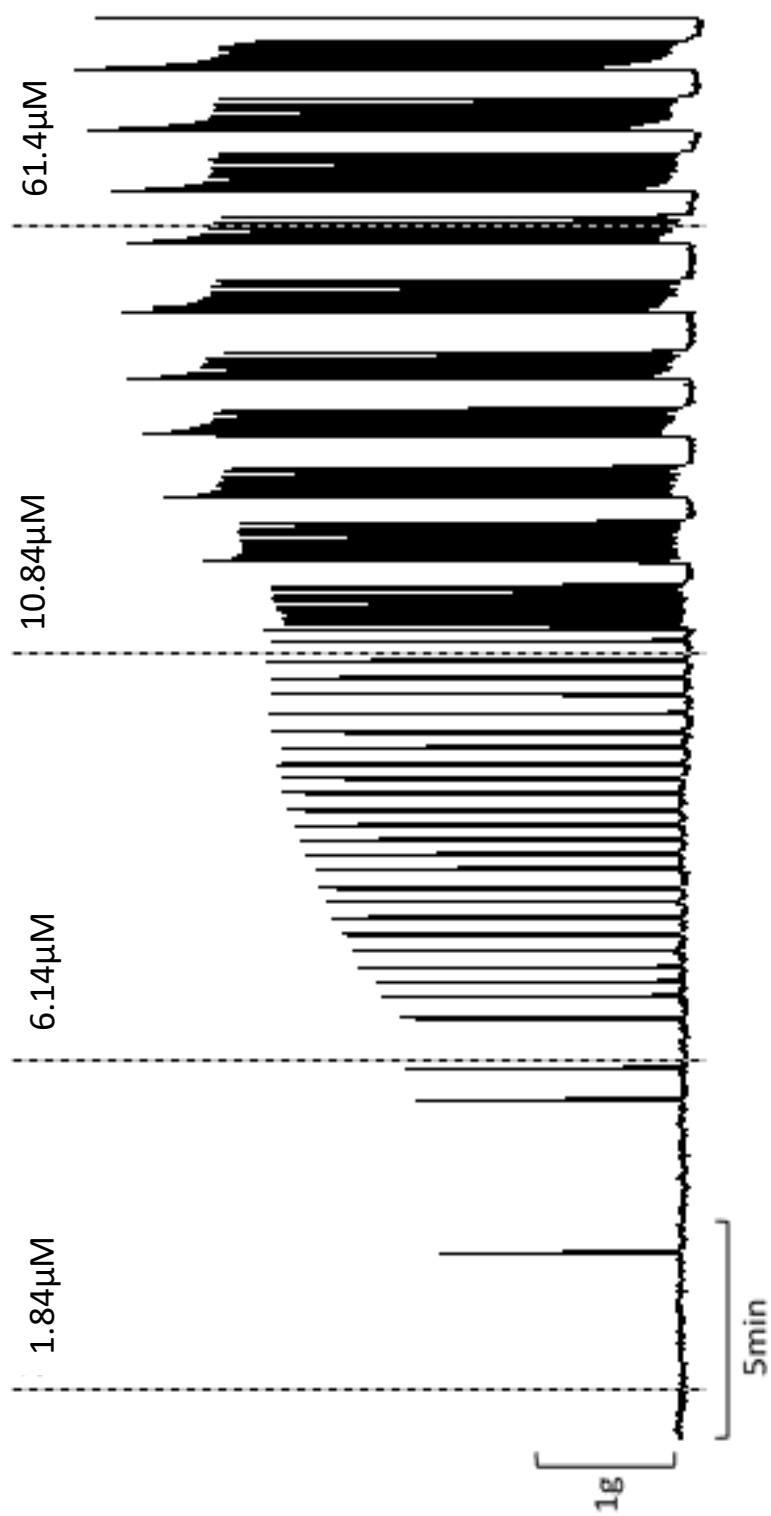


Figure 2.2 Example isometric force recording showing phasic contractions developed by pig isolated distal ureter to cumulative concentrations of phenylephrine (1.84-61.4μM). Vertical bar indicates tension (g) and horizontal bar indicates time (min).

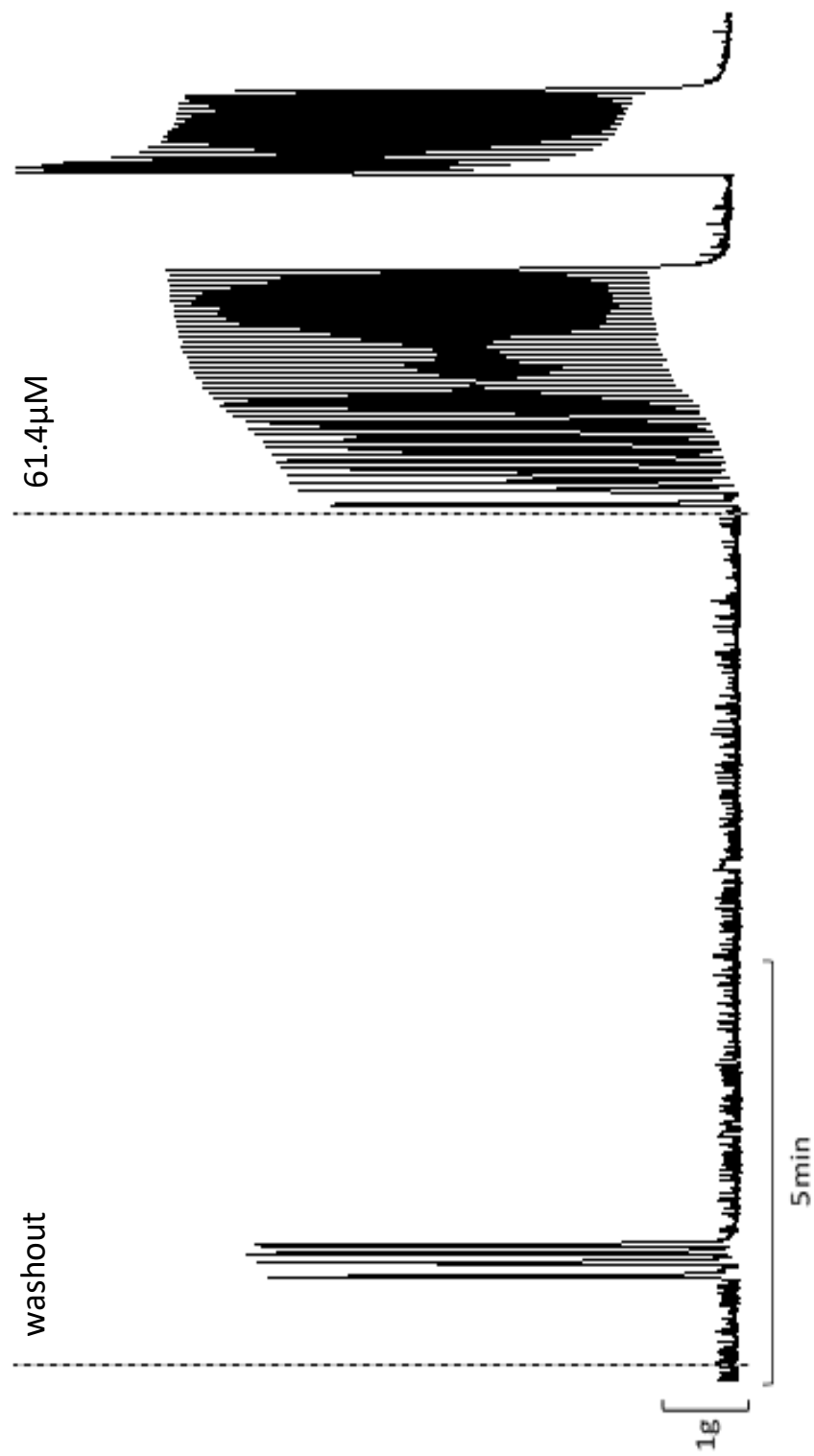
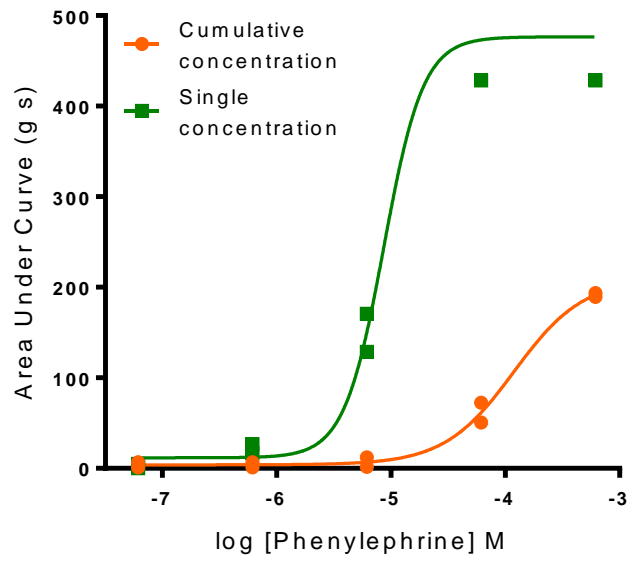


Figure 2.3 Example isometric force recording showing phasic contractions developed in pig isolated distal ureter in response to a single concentration of phenylephrine (61.4μM). Vertical bar indicates tension (g) and horizontal bar indicates time (min).

(a)



(b)

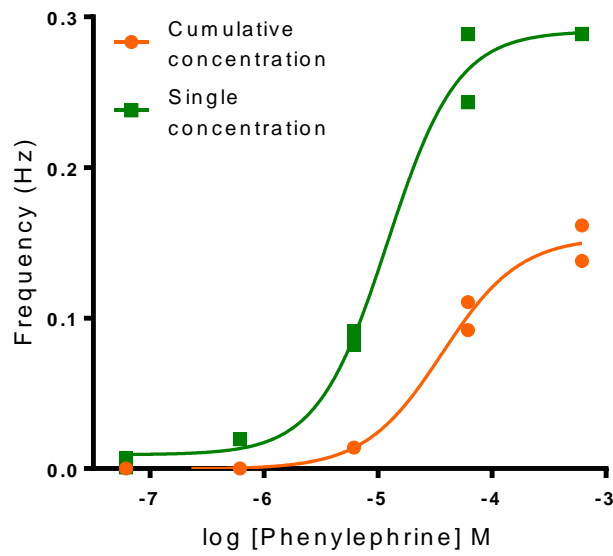


Figure 2.4 Comparison of responses of pig distal ureter following cumulative vs single concentrations of phenylephrine. Responses are expressed as AUC in g s (a) and frequency in Hz (b).

2.3 Optimization of Tissue Strip Orientation

Introduction

The technique of *in vitro* isolated tissues is a useful and important method to investigate the physiology and pharmacology of various animal and human tissues, including the ureter. However, in previous studies of the *in vitro* function of isolated ureter, different laboratories have utilised various methods for preparing and mounting ureteral tissues. Thus, this prevents a valid comparison of data.

As discussed in the Chapter 1, transverse sections of ureter have been studied previously and it has been shown that muscular bundles occur in two layers within the ureter, the outer muscular bundles that are orientated in a circular manner and the inner orientated longitudinally (Hanna et al., 1976). Due to these findings, some reports suggest that a spiral-cut segment of the ureter preserves more continuous muscular bundles and can generate more powerful spontaneous contractions (Morita et al., 1995). Despite this proposition, in a previous study, intact ring segments, open longitudinal tubes, closed longitudinal tubes and open spiral cut segments of porcine isolated ureter, as shown in Figure 2.5, were compared and it was found that responses did not vary (Jerde et al., 1999). Therefore, most studies have utilised the open longitudinal tissue preparation, as spiral preparations utilise a greater amount of tissue and are not ideal for studies involving limited human or animal tissues.

However, a circular preparation, as shown in Figure 2.6, has not been previously examined. Therefore, as part of the methods optimization, the aim was to compare the contractile responses between the following two types of ureteral preparations, longitudinal and circular, to determine which method was most optimal for the study of agonist-mediated contractile responses.

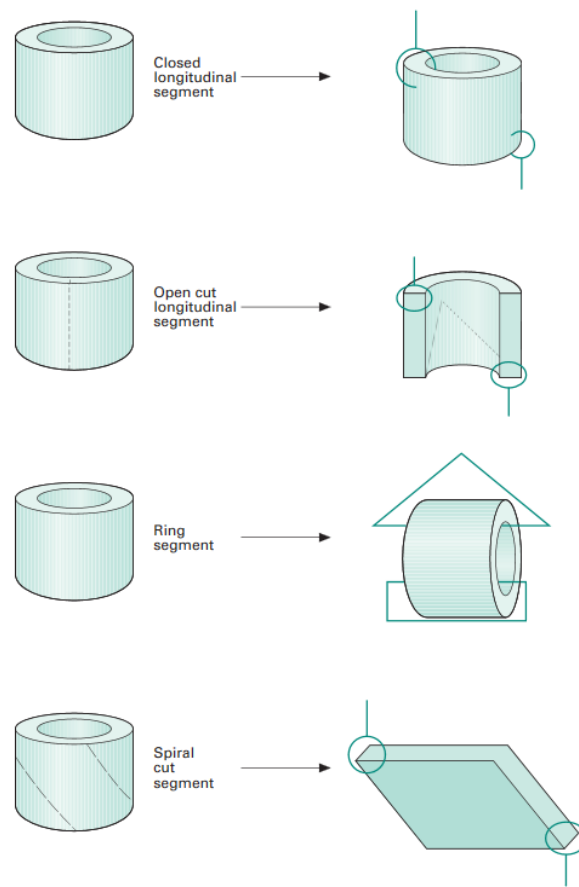


Figure 2.5 Preparations of ureteral tissues used in previous studies (Jerde et al., 1999).

Methods and Materials

Tissue preparation and *in vitro* functional studies were performed as stated in Section 2.1. The tissue strip orientations that were compared in this study are longitudinal and circular preparations and these were dissected and prepared as shown in Figure 2.6. The agonist used in this study was phenylephrine ($7.48 \times 10^{-8} - 7.48 \times 10^{-4}$ M). Adjacent tissue strips were set-up in adjacent organ baths for comparison of contractile responses between circular and longitudinal segments. Contractile responses were expressed as AUC (g s) and frequency (Hz).

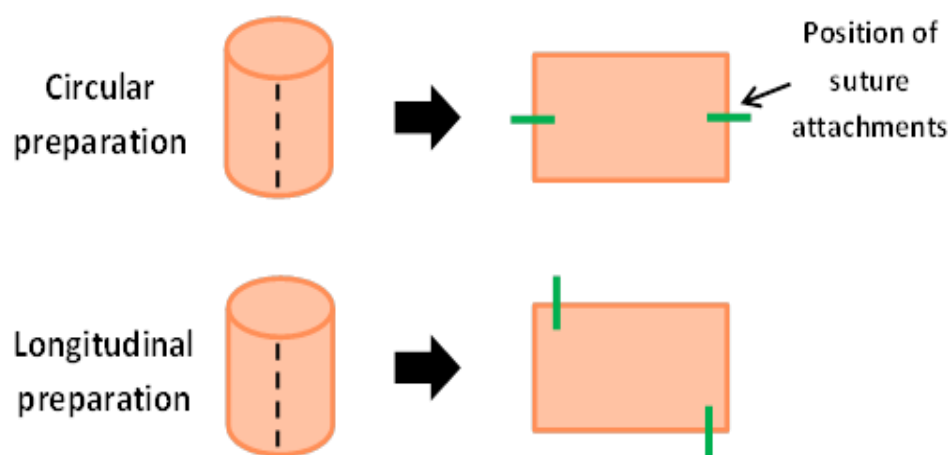


Figure 2.6 The two different methods used for preparation of ureteral segments. The lumen of ureteral segments was dissected open and attachments of suture for recording were positioned longitudinally (longitudinal preparation) or transversely (circular preparation) across the tissue strip.

Results

All porcine ureteral strips were allowed to equilibrate to a passive tension of $1.05\text{g} \pm 0.04\text{g}$ ($n=8$). Spontaneous contractions developed in 3 of 8 ureteral strips. When quiescent tissue strips were subjected to increasing concentrations of phenylephrine, both longitudinal and circular segments of porcine ureter developed bursts of phasic contractions. Also, increasing concentrations of phenylephrine induced increases in frequency of phasic activity in all porcine distal ureteral strips. However, the amplitude of phasic activity was not concentration-dependent.

The potency (pEC_{50}) of phenylephrine in the longitudinal segments was significantly greater than in the circular segments ($p<0.05$, paired t -test, $n=4$, Table 2.3). In addition, the maximum AUC of the contractile responses was also greater in the longitudinal strips ($p<0.05$, paired t -test,

n=4, Fig 2.7a). Maximum frequency was also greater in longitudinal segments ($p<0.05$, paired t -test, n=4, Fig 2.7b).

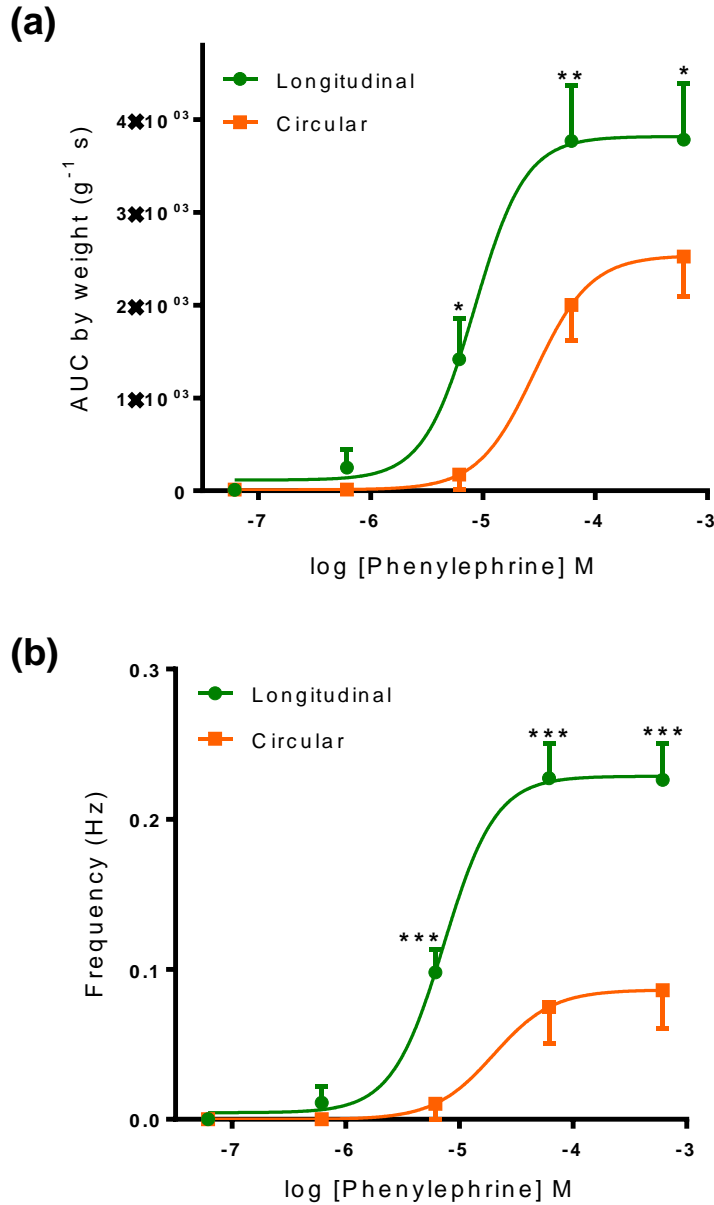


Figure 2.7 Concentration-response curves for phenylephrine in the distal pig ureter in longitudinal and circular preparations. Responses are expressed as AUC by tissue weight (a) and frequency (b). Data are presented as mean \pm SEM of 4 preparations in each group. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs circular)

Table 2.3 *pEC₅₀ values for AUC for phenylephrine responses in the pig ureter. Data are expressed as mean \pm SEM (* vs circular segments).*

Orientation	n	pEC ₅₀
Longitudinal	4	5.08 \pm 0.27*
Circular	4	4.54 \pm 0.22

Discussion

A previous study reported that different preparations of pig ureteral tissue, including open longitudinal (similar to the longitudinal preparations in our study), closed longitudinal, closed ring and spiral cut segments all produced contractile responses that were similar in spontaneous contraction rates and maximum amplitude, in response to electrical field stimulation (EFS) and muscarinic stimulation with carbachol (Jerde et al., 1999). However, no studies have investigated the open circular segment utilised in the present study.

The longitudinal smooth muscle layer in the ureter is responsible for the shortening of the ureter while the circular smooth muscle contracts to generate lumen pressure and coapting of the ureteral walls. The present results indicate that the potency of phenylephrine, maximal AUC responses and maximal frequency are significantly greater in the longitudinal segments compared to the circular segments of ureter. This could be due to the greater amount of longitudinal smooth muscle compared to circular smooth muscle. However, there is no histological data on quantification of different smooth muscle types of the ureter available currently in the literature. Additionally, another explanation for

the results obtained could be that longitudinal smooth muscles cells of the ureter are more powerful compared to the circular smooth muscle cells and therefore were able to produce greater contractile responses. A recent mechanical study on the human ureter smooth muscle showed that the longitudinal smooth muscle possessed a significantly higher rate of myosin cross bridge interactions in comparison to the circular layer (Vargiu et al., 2015). Therefore, this could likely result in a stronger contraction within the longitudinal layer of smooth muscle in comparison to the circular layer.

It has been suggested that an increase in contractility and stretching of both circular and longitudinal smooth muscles surrounding a stone significantly amplifies pain, causing ureteral colic (Rose and Gillenwater, 1973). Most studies on the isolated ureter *in vitro* utilise longitudinal tissue preparations. In light of these results, longitudinal segments were used in all subsequent experiments in this thesis. This also allowed for a more reliable comparison between reported results of ureteric study in the literature and our results.

2.4 Reproducibility of Concentration-Response Curves

Introduction

The desensitization of isolated ureteral smooth muscle strips was observed in preliminary experiments to cumulative concentrations of agonists. In addition to this, there have been numerous reports of desensitization of agonist-mediated responses in the urinary tract. In order to investigate the effects of antagonists on responses of isolated ureteral smooth muscle, we aimed to determine the reproducibility of concentration-response curves to various agonists.

Methods and Materials

Tissue preparation and *in vitro* functional studies were performed as stated in Section 2.1. The agonist used in this study was phenylephrine (7.48×10^{-8} – 7.48×10^{-4} M). Tissue strips were subjected to increasing concentrations of phenylephrine and washed with Krebs solution until contractile responses returned to baseline, before the strips were subjected to increasing single concentrations of phenylephrine again. Contractile responses were expressed as a percentage of the maximal response in AUC ($\text{g}^{-1} \text{ s}$) and frequency (Hz).

Results

All porcine ureteral strips were allowed to equilibrate to a passive tension of $1.18\text{g} \pm 0.09\text{g}$ ($n=6$). No spontaneous contractions developed in any tissues strips. Increasing concentrations of phenylephrine triggered bursts of phasic contractions in quiescent tissue strips. Increasing single

concentrations of phenylephrine induced increases in frequency of phasic activity in all porcine distal ureteral strips, while amplitude of phasic activity was not concentration-dependent.

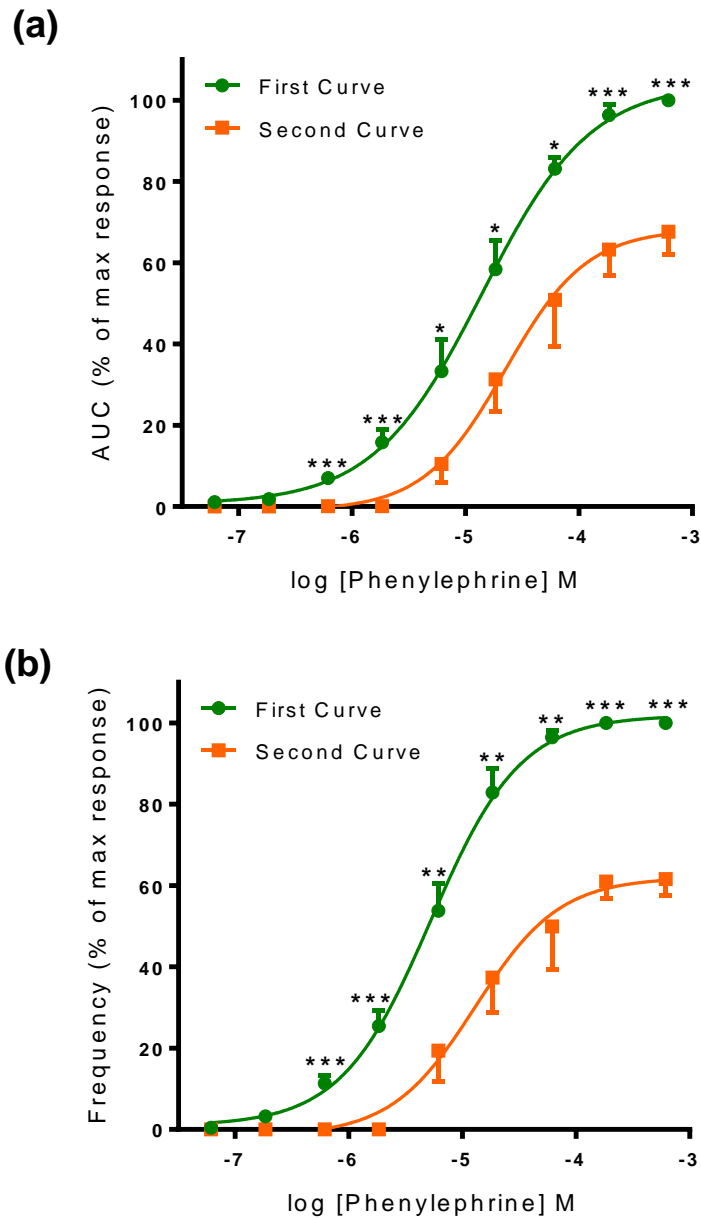


Figure 2.8 Comparison of first and second concentration-response curves for phenylephrine in the distal pig ureter. Responses are expressed as percentage of the maximal AUC (a) and frequency (b). Results are presented as mean \pm SEM of 6 preparations. (* p <0.05, ** p <0.01, *** p <0.001 vs second curve)

Table 2.4 <i>pEC₅₀ values for AUC of phenylephrine responses in the pig ureter. Results are expressed as mean \pm SEM.</i>		
Curve	n	pEC₅₀
First	6	4.85 \pm 0.07
Second	6	4.65 \pm 0.12

The potency (pEC₅₀) of phenylephrine was similar for first and second curves (Table 2.4). Maximum AUC of the contractile responses was reduced by 33% in the second curve ($p < 0.001$, paired t -test, $n=6$, Figure 2.8a). Maximum frequency was also depressed by 40% in the second phenylephrine concentration-response curve ($p < 0.001$, paired t -test, $n=6$, Figure 2.8b). This reduction was not only observed at maximal concentrations of phenylephrine but also at submaximal concentrations.

Discussion

Our findings have shown that maximum contractile responses expressed as AUC and frequency were diminished in the subsequent phenylephrine concentration-response curve (Figure 2.8). The inability of the same isolated pig ureteral strip to contract to its full potential for the second time to increasing concentrations of phenylephrine is likely due to the desensitization of the α_1 -adrenoceptors in the smooth muscle cells.

Receptor desensitization occurs when a diminished response to an agonist is obtained when compared to the initial response (Collins et al., 1990). This is usually considered as a pharmacological event caused by prolonged stimulation by an agonist or by stimulation with a high agonist concentration. It is thought that in GPCRs like α_1 -adrenoceptors, a self-

limited signal is produced when exposed to continuous stimulation by an agonist (Ferguson et al., 1998). Studies have shown that desensitization can involve various processes including changes in cellular receptor compartmentalization, receptor number and downstream signal transduction machinery (Thompson et al., 2005). In a previous study, it was reported that among three human α -adrenoceptor subtypes, only α_{1A} - and α_{1B} -adrenoceptors were downregulated with prolonged phenylephrine exposure while α_{1D} -adrenoceptors were in fact, upregulated (Yang et al., 1999). These effects were observed as soon as 2 hours of phenylephrine exposure (Yang et al., 1999). In this same study, it was also observed that these treatment conditions also caused a downregulation in inositol phosphate formation via α_{1A} - and α_{1B} -adrenoceptors but not in α_{1D} -adrenoceptors (Yang et al., 1999). However, mRNA expression was reduced only for α_{1B} -adrenoceptors and not for α_{1A} -adrenoceptors upon phenylephrine treatment, suggesting that desensitization of α_{1A} -adrenoceptor may occur independent of changes in corresponding mRNA (Yang et al., 1999). In the human and pig isolated ureter, it was found that although expression of α_{1D} -adrenoceptor subtype is the greatest (Itoh et al., 2007), the subtype that plays the dominant functional role in smooth muscle contraction is α_{1A} -adrenoceptor and not other adrenoceptor subtypes (Sasaki et al., 2011). Therefore, it is most likely that the desensitization observed in the present study is due to downregulation of inositol phosphate formation via α_{1A} -adrenoceptors over prolonged exposure to agonist phenylephrine. While further molecular studies are required, alteration in mRNA expression of adrenoceptors is less likely.

In conclusion, the depressed response in the smooth muscle cells of the ureter upon phenylephrine treatment in the present study could be due to α_{1A} -adrenoceptor desensitization. For this reason, in all further experiments, paired adjacent ureteral tissue strips were utilised and only one concentration-response curve was constructed on each tissue, to avoid the variable of desensitization in antagonist experiments.

2.5 Effect of Age on Ureteral Responses

Introduction

Ageing has been shown to be a risk factor for urinary stone formation and is associated with declining function in almost every physiological system (Hess, 2003). Thus, it is vital to investigate how physiological function alters as we age, to aid in drug discovery and treatment of disease in the ageing society. However, it is often difficult to distinguish between dysfunction caused by disease and modifications that are solely age-related.

In the lower urinary tract, there have been many studies that have investigated how bladder function and neural activity are affected by ageing. Morphological studies demonstrate that there is an age-dependent decrease in numbers of unmyelinated afferents innervating the bladder, but the general innervation pattern seems to be preserved in rats (Aizawa et al., 2011, Mohammed and Santer, 2002, Nakayama et al., 1998). In addition to this, functional and histological investigations have produced contradicting results, where some found a decrease in detrusor muscle thickness, collagen deposition and contractility with age in the rat bladder (Lluel et al., 2000) while others have reported no change in the mouse bladder (Smith et al., 2012).

Very little research has been directed towards the effects that ageing has on the normal functioning system of the ureter. α_1 -adrenoceptor-mediated contractions in the ureteral smooth muscle have been described in human (Park et al., 2007), canine (Wanajo et al., 2005), mouse and hamster (Kobayashi et al., 2009). It has been shown that the receptor subtype responsible for contraction to noradrenaline is the α_{1A} -adrenoceptor in human ureteral tissues, but the most abundant subtype at the mRNA level is the α_{1D} -adrenoceptor (Sasaki et al., 2011). Although no studies have

investigated α_1 -adrenoceptor-mediated responses in ureter with age, age-related changes in α -adrenoceptor-mediated responses have been investigated in other tissues of the lower urinary tract, where increased contractile responses to phenylephrine were observed in the rat bladder with age (Ordway et al., 1986b). In other systems besides the urinary tract, ageing has also been shown to produce changes in α -adrenoceptor responses and these include the rat vas deferens (Yono et al., 2008, Docherty and O'Malley, 1983), rat isolated trachea (Preuss et al., 1998), and vascular preparations from rats (Docherty, 1988, Docherty and O'Malley, 1983) and dog (Toda and Shimizu, 1987). In contrast, it has been reported that there was no age-related change in α -adrenoceptor-mediated response in human vascular tissue (Stevens et al., 1982, Scott and Reid, 1982) or rat tail arteries (Tsai et al., 1993).

Although it has been shown that the α -adrenergic system plays the major role in contractility of the ureter, some studies have demonstrated the distribution of acetylcholinesterase-positive fibres at the distal ureter (Schulman, 1975, Prieto et al., 1990). Additionally, stimulation by muscarinic agonists has been shown to induce contractile responses in the isolated ureter of various species including pig (Hernandez et al., 1993), dog (Tomiyama et al., 2003b) and horse (Prieto et al., 1994). In the bladder, the effects of ageing on contractile responses induced by muscarinic agents are contradictory (Munro and Wendt, 1993, Lieu et al., 1997, Ordway et al., 1986a). Nevertheless, changes were observed with ageing and this suggests that modifications with age in muscarinic receptors could also occur in the ureter.

5-hydroxytyptamine (5-HT) has been shown to have a variety of actions on many cell types including smooth muscle cells. Pharmacological studies have shown that 5-HT evokes contractions in the ureter *in vivo* (Fetisssof et al., 1983) and *in vitro* (Hernandez et al., 2003, Gidener et al., 1999,

Gidener et al., 1995). In one study, the detrusor muscle was shown to have altered contractile responses to 5-HT with ageing (Saito et al., 1993).

Histamine is an inflammatory mediator released by basophils and mast cells that could be involved in the mediation of smooth muscle contractions. Although very little research has been directed at histamine-stimulated responses in the ureter, studies on the human and dog ureter have shown that contraction was induced by histamine (Bertaccini et al., 1983, Dodel et al., 1996). There was no effect of age on histamine-induced contractions in the male rat bladder (Chun et al., 1989) but contractions were greater in the young rabbit in comparison to the older group (Zderic et al., 1990).

In our preliminary experiments, we noticed different responses in tissues from two different age groups of pigs available at the abattoir (old: 24 months, young: 3 months). Therefore, it was of interest to compare the responses of the ureters between the age groups. The aim of the present study was to investigate the effect of age on smooth muscle contractility in the porcine ureter.

Methods and Materials

Tissue preparation and *in vitro* functional studies were performed as stated in Section 2.1. The agonists in this study were phenylephrine (7.48×10^{-8} – 7.48×10^{-4} M), carbachol (6.84×10^{-8} – 6.84×10^{-4} M), 5-HT (5.88×10^{-8} – 5.88×10^{-4} M), and histamine (1.12×10^{-7} – 1.12×10^{-3} M). Contractions to agonists were expressed as AUC by weight ($\text{g}^{-1} \text{ s}$) and frequency (Hz).

Results

The ureteral strips were allowed to equilibrate at a passive tension of $1.01\text{g} \pm 0.12\text{g}$ ($n=56$) and spontaneous contractions developed during equilibration period in 16 of 56 (29%) ureteral strips. When subjected to increasing single concentrations of phenylephrine and 5-HT, porcine ureteral tissues from both age groups developed bursts of phasic contractions and increasing concentrations caused increased frequency of phasic activity (Figures 2.9 and 2.11). In the presence of increasing concentrations of carbachol, only isolated ureteral strips from the older age group produced contractile responses, which were of a similar pattern (Figure 2.10). Increasing concentrations also resulted in an increase of frequency in phasic activity. No ureteral strips responded to stimulation by histamine (not shown).

The potency (pEC_{50}) of agonists was similar for tissues from both age groups, where comparison was possible (phenylephrine and 5-HT) (Table 2.5). However, the maximum contractile responses to phenylephrine expressed in AUC ($p<0.001$, unpaired t -test, $n=8$, Figure 2.9a) and frequency were significantly enhanced in tissues from the older group ($p<0.05$, unpaired t -test, $n=8$, Figure 2.9b). In contrast, for 5-HT, maximum AUC responses ($p<0.001$, unpaired t -test, $n=8$, Figure 2.11a) were depressed in tissues from older animals. However, the maximum frequency was similar in tissues from both age groups (Figure 2.11b).

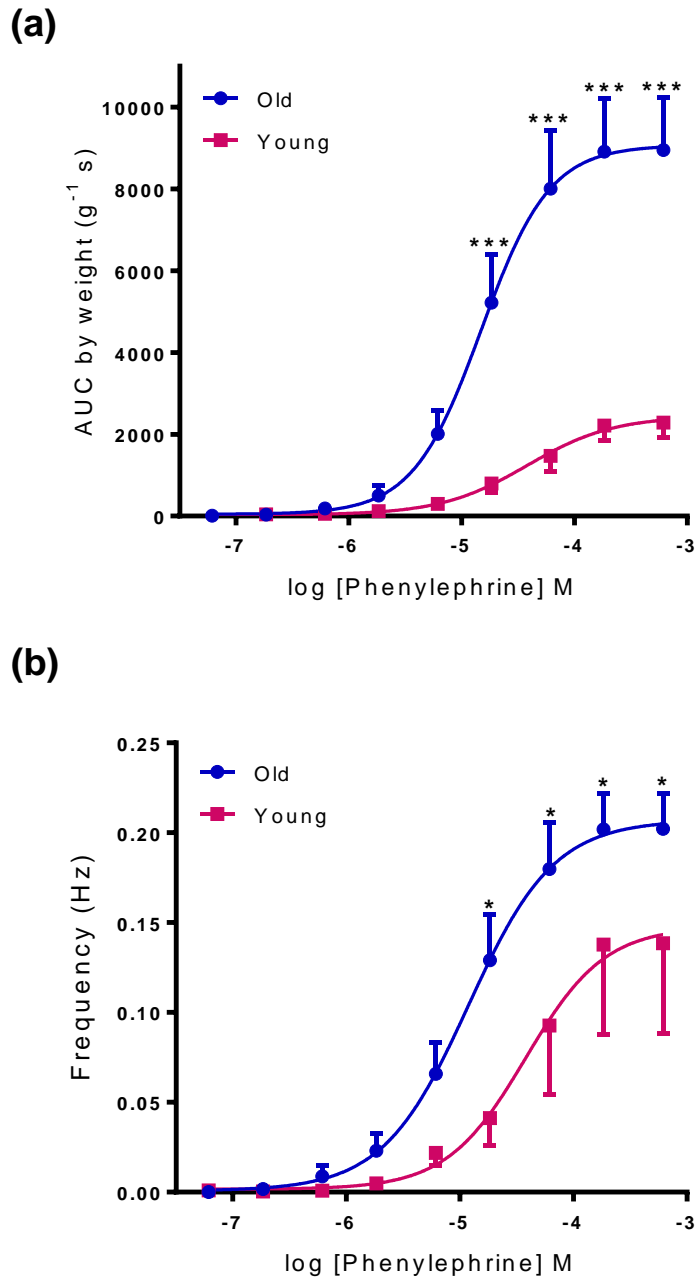


Figure 2.9 Concentration-response curves for phenylephrine in distal ureteral tissues from old and young pigs. Responses are expressed as AUC by weight in g s (a) and frequency in Hz (b). Results are presented as mean \pm SEM of 8 preparations for each (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs young)

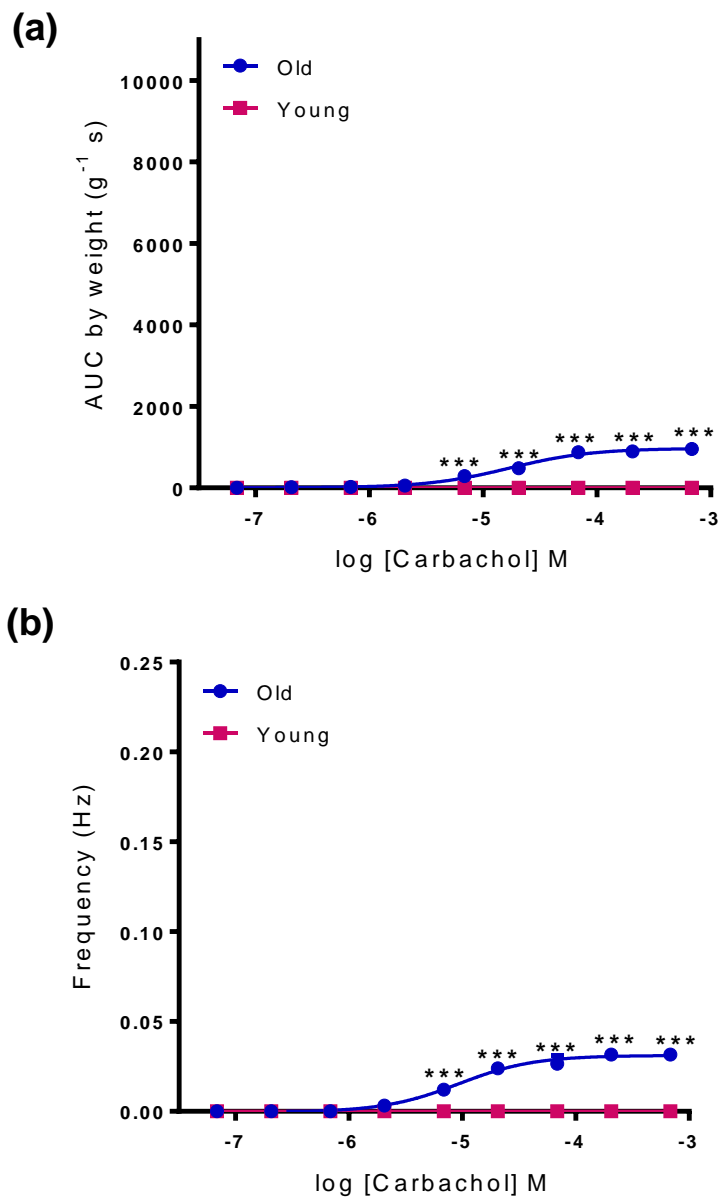


Figure 2.10 Concentration-response curves for carbachol in distal ureteral tissues from old and young pigs. Responses are expressed as AUC by weight in g s (a) and frequency in Hz (b). Results are presented as mean \pm SEM of 8 preparations for each group (* p <0.05, ** p <0.01, *** p <0.001 vs young)

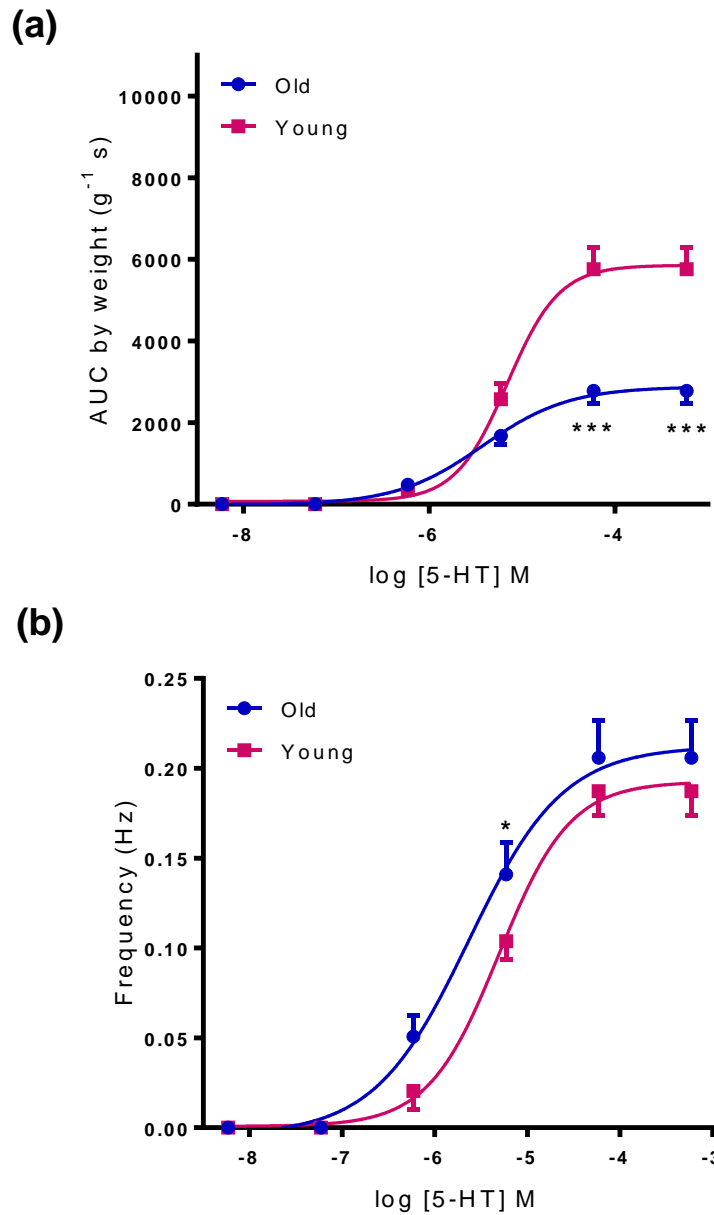


Figure 2.11 Concentration-response curves for carbachol in distal ureteral tissues from old and young pigs. Responses are expressed as AUC by weight in g s (a) and frequency in Hz (b). Results are presented as mean \pm SEM of 8 preparations for each group. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs young)

Table 2.5 *pEC₅₀ values for AUC in responses to various agonists in the distal ureters from old and young pigs. Results are expressed as mean \pm SEM.*

Agonist	Group	n	pEC ₅₀
Phenylephrine	Young	8	4.42 \pm 0.18
	Old	8	4.83 \pm 1.43
Carbachol	Young	8	-
	Old	8	4.77 \pm 0.11
5-HT	Young	8	5.16 \pm 0.09
	Old	8	5.43 \pm 0.16

Discussion

In the present study, it was observed that isolated porcine ureteral smooth muscle demonstrates age-related changes in contractions in response to α -adrenoceptor, muscarinic and 5-HT receptor stimulation. Maximal contractile responses to the α -adrenoceptor agonist phenylephrine and the muscarinic receptor agonist carbachol were greater in older animals, whereas contractile responses to 5-HT were greater in younger animals. There were no changes in potency for any of the agonists, where possible comparison was permitted (phenylephrine and 5-HT). Therefore, changes observed in the maximal contractions are unlikely to be caused by an increase or decrease in the number of functional receptors expressed with age or affinity of the receptors for the agonist, as a decrease in receptor expression should produce a rightward shift of concentration curves. Additionally, it is also unlikely that these age-related changes are due to a general modification of smooth muscle contraction capability with age, as

phenylephrine and carbachol induced greater contractions with age whilst 5-HT-stimulated contractions were reduced with age.

Increase in Phenylephrine-Induced Contractile Responses with Age

The findings with regards to α -adrenoceptor stimulation in the ureter support previous findings demonstrating an increase in contractile responses to norepinephrine with age in the isolated rat urinary bladder, where no change in potency was observed with age (Saito et al., 1993, Ordway et al., 1986b). Likewise, it was found that norepinephrine-induced contractile responses were greater in the bladders of older rats (Ordway et al., 1986b). Besides the lower urinary tract, studies on the effect of ageing on α -adrenoceptor-mediated contractile responses in other physiological systems have also been shown. Isolated rat tracheal strips elicited increased maximal contractions to noradrenaline in older animals, where pEC_{50} values were unaffected (Preuss et al., 1998). In this same study, ageing did not affect maximal contraction or pEC_{50} values for noradrenaline in isolated trachea from guinea-pig, indicating that age-dependent α -adrenoceptor stimulation may be species-dependent (Preuss et al., 1998).

In the human and porcine ureter, the dominant functional α_1 -adrenoceptor subtype is the α_{1A} -adrenoceptor subtype (Sasaki et al., 2011). To our knowledge, there are currently no studies on the effect of ageing ureteral contractile responses. While mRNA studies of the various α -adrenoceptor subtypes have shown that there is a possible change of receptor subtype expression dominance from the α_{1A} -adrenoceptor subtype to α_{1B} -adrenoceptor with maturation in aortic tissues of rats (Gurdal et al., 1995), this is unlikely to be the case to explain the present findings in the pig ureter. A change in receptor number might be evident in the case where maximal response to a partial agonist is altered or pEC_{50} values for full agonist are changed. However, the present results show that the pEC_{50}

value for phenylephrine, a full agonist, was not altered with age, which suggests that the increased contractile responses are not due to modification of α_{1A} -adrenoceptor expression, but a change in the efficacy of the agonist. Alteration of receptor efficacy could be due to changes in the intracellular signalling pathway/s resulting in the contractile responses.

It is thought that the preferential intracellular pathway of α_1 -adrenoceptors is via G_q subunit coupling, activating the phospholipase C pathway which elevates levels of inositol (1,4,5)-triphosphate and intracellular Ca^{2+} , and releasing diacylglycerol that in turn allows protein kinase C activation. In addition to this, it has been reported that activation of α_1 -adrenoceptors also caused the activation of phospholipase A_2 , phospholipase D, adenylate cyclase and mitogen-activated protein kinases (Perez et al., 1993, Graham et al., 1996, Zhong and Minneman, 1999, Piascik and Perez, 2001). The discovery of these multiple signalling pathways produces the possibility for activation of one subtype of α_1 -adrenoceptor producing different outcomes in the cell. In an investigation measuring the capacity of α_{1A} -adrenoceptor stimulation to activate various signalling pathways, phenylephrine showed significant bias towards extracellular acidification rate versus Ca^{2+} release and cAMP accumulation (Evans et al., 2011). While there are currently no studies to our knowledge that have investigated these intracellular signalling pathways in the ureter, the possibility of an increase in extracellular acidification rate in the isolated porcine ureter with age alteration in functional intracellular cellular pathways may explain the increase in contractile response to phenylephrine with age in the present study.

Increase in Carbachol-Induced Contractile Responses with Age

In the bladder, contractions in response to cholinergic receptor stimulation by acetylcholine in denuded detrusor muscle strips were enhanced in tissues from aged mice (Daly et al., 2014) and older rats (Longhurst et al.,

1992, Ordway et al., 1986a). The present study supports these findings in the lower urinary tract given that isolated ureters from younger animals did not respond to carbachol, whereas tissues from older animals did.

While no molecular studies have been performed on muscarinic receptors in the ureter, a previous study suggested that the M₁ receptor subtype regulated tonic responses in the pig intravesical ureter and phasic responses involved M₂, M₃ and M₄ muscarinic receptors (Hernandez et al., 1993). However, the age group of the animals used in the stated study was not reported and therefore, it is difficult to draw conclusions about expression with age. In the present study, it was observed that there is no phasic nor tonic response to carbachol in isolated ureter from younger animals, suggesting the possibility that these muscarinic receptor subtypes may not be expressed before a certain age in the ureter. However, most studies on the lower urinary tract, particularly the bladder, suggest a decrease in muscarinic receptor expression with age. A study using quantitative competitive RT-PCR demonstrated a selective age-related decrease in mRNA for muscarinic M₃ receptors in detrusor of human bladder (Mansfield et al., 2005). However, RT-PCR showed that expression of mRNA of M₂ and M₃ muscarinic receptor subtypes were similar in isolated detrusor from juvenile and old pigs (Wuest et al., 2008). Thus the effect of age in the lower urinary tract is still not clear, and while it is not possible to directly compare the bladder with the ureter, these reports present the potential for a change in muscarinic receptor-mediated expression with age in the porcine isolated ureter. It is also possible that alterations in intracellular signalling pathways contribute to these changes.

Decrease in 5-HT-Induced Contractile Responses with Age

Whilst a few studies have examined the effects of ageing on responses of smooth muscle within lower urinary tract to 5-HT, results have been inconsistent. In studies of the rat urinary bladder, *in vitro* detrusor strips

have shown an increase in contractile response to 5-HT with age, while another study showed that responses were unaffected (Saito et al., 1993, Chun et al., 1989). Outside of the lower urinary tract, generally age does not appear to affect smooth muscle responses to 5-HT. Contractile responses to 5-HT in the guinea-pig and rat isolated trachea were shown not to differ in potency or maximal response between age groups (Cox and Cohen, 1994, Preuss and Goldie, 1998). Similarly, changes observed in rat vascular contractions to α -adrenoceptor stimulation due to age were not observed for 5-HT-mediated contractile responses (Cai et al., 1994). Although most studies of smooth muscle indicate that contractile activity to 5-HT is not dependent on age, a similar finding to the present study was observed in the human basilar artery, where maximal contractions stimulated by 5-HT decreased with age (Hatake et al., 1992).

Similar to pEC_{50} values of phenylephrine, pEC_{50} values for 5-HT were also unaffected by age. Because 5-HT is a full agonist for its receptors, it is also unlikely that the decrease in maximal 5-HT-induced contraction with age is due to changes in receptor expression but instead is due to altered efficacy of receptor stimulation. It is suggested that the differences in 5-HT-stimulated contractile responses with age presented here could be due to alterations in the involvement of other receptor subtypes to contractility with age. This is further discussed in the following chapter, where the 5-HT receptor subtype/s responsible for 5-HT-stimulated contractile responses were pharmacologically characterised in ureteral tissues from old and young animals (Chapter 3).

Summary

In conclusion, contractile responses mediated by adrenergic, muscarinic and 5-HT receptors are altered with age in the porcine ureter. It is suggested that these age-related modifications in adrenergic responses may be due to changes in the intracellular signalling pathways stimulated

by receptor activation, while in muscarinic receptor mediated contractions, the alterations may be due to changes in receptor expression. However, further molecular and pharmacological studies are required. With regard to 5-HT-mediated contractions, the following chapter investigates in more detail the possible cause for age-related changes in responses to 5-HT.

For the purpose of this thesis, these findings were crucial, as it is clear that animal age must be a consideration when studying the pharmacology of smooth muscle tissues, including the ureter. All subsequent experiments in this thesis were performed in both age groups to determine whether there were any further age-related changes where all figures for tissues from young animals are presented in **pink** and figures for tissues from old animals in **blue**.

2.6 Regional Difference in Responses of the Ureter

Introduction

The ureter can be divided into three regions: the proximal, mid and distal ureter. The proximal ureter is the part closer to the kidney and the distal ureter is the lower part approaching the bladder. Studies have shown that the most common location for lodgement of ureteral calculi is in the distal ureter, approaching the ureterovesical junction, where the ureter enters the bladder, with this region accounting for 54% of stones requiring surgical intervention (El-Barky et al., 2014). The second most common location, accounting for 34% of stones, is just below the pelvi-ureteric junction where the proximal ureters leave the kidneys (El-Barky et al., 2014).

It is proposed that electrical activity arises at the renal pelvis where atypical smooth muscle cells act as the primary pacemakers and conduct peristaltic waves to the ureter (McHale et al., 2006). In addition to this, studies have shown that the innervation density of the autonomic nervous system progressively increases from the renal pelvis to the bladder, and therefore the distal ureter will be more richly innervated than the proximal (Edyvane et al., 1992). The purpose of this study was to compare 5-HT and phenylephrine-induced contractile activity in the two different regions of the pig isolated ureter.

Methods and Materials

Tissue preparation and *in vitro* functional studies were performed as stated in Section 2.1. The proximal region of the ureter close to the kidneys and the distal region close to the bladder were isolated. The

agonists used in this study were phenylephrine (7.48×10^{-8} – 7.48×10^{-4} M) and 5-HT (5.88×10^{-8} – 5.88×10^{-4} M). Contractions to agonists were expressed as percentage of maximal response in AUC (g s) and frequency (Hz).

Results

Porcine ureteral strips equilibrated to a passive tension of 0.98 ± 0.09 (n=46). Spontaneous contractions developed during the equilibration time in 20 of 23 proximal ureteral strips (87%) and 6 of 23 distal ureteral strips (26%).

The potency (pEC_{50}) of agonists was similar between tissues from the two different regions and between the tissues from young and old animals (Table 2.6). While maximum AUC to phenylephrine was similar (Figure 2.12a) in tissues from the two regions in the young group, maximum frequency was greater in the proximal strips from young animals compared to distal ($p < 0.005$, paired *t*-test, n=6, Figure 2.12b). In contrast, in the older animals, maximum contractile responses to phenylephrine expressed as AUC were significantly greater in the distal region of the isolated ureter compared to proximal ($p < 0.05$, paired *t*-test, n=5, Figure 2.13a), although maximum frequency was similar in both regions (Figure 2.13b).

Maximum contractile responses to 5-HT expressed as AUC were smaller in the proximal region than in the distal region in both age groups ($p < 0.001$, paired *t*-test, n=6, Figures 2.14a and 2.15a). Maximum frequency in response to 5-HT was not different between the proximal and distal regions in tissues from both age groups (Figures 2.14b and 2.15b).

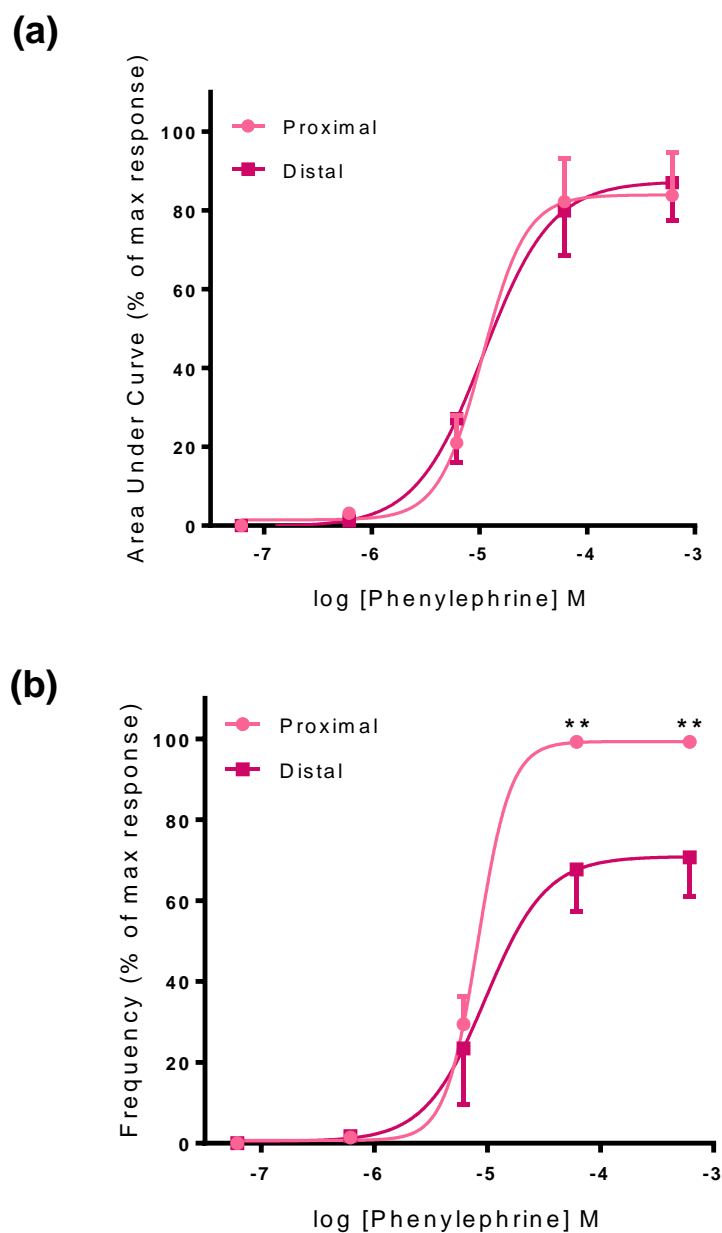
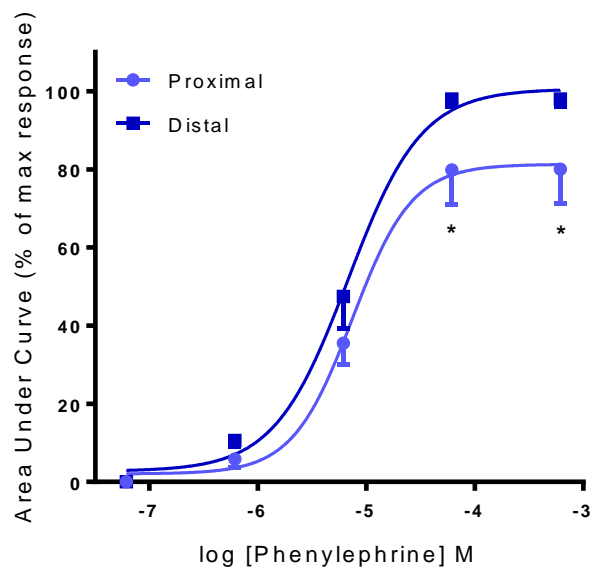


Figure 2.12 Concentration-response curves for phenylephrine in the proximal and distal ureter from young pigs. Responses are expressed as percentage of maximal AUC (a) and frequency (b). Results are presented as mean \pm SEM (* p <0.05, ** p <0.01, *** p <0.001 vs distal) of 6 preparations for each group.

(a)



(b)

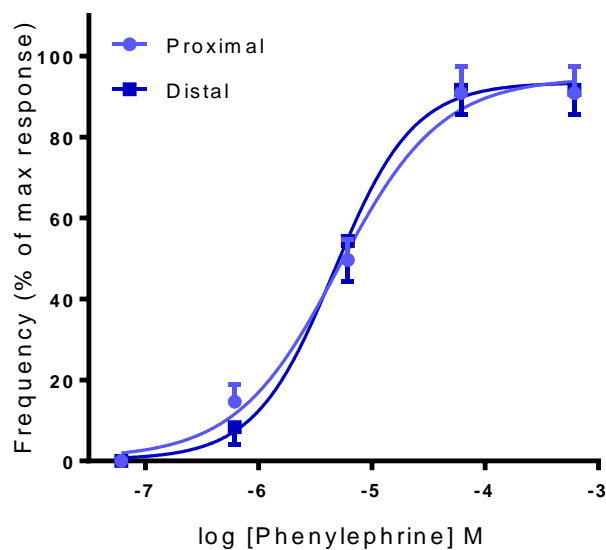


Figure 2.13 Concentration-response curves for phenylephrine in the proximal and distal ureter from old pigs. Responses are expressed as percentage of maximal AUC (a) and frequency (b). Results are presented as mean \pm SEM (* p <0.05, ** p <0.01, *** p <0.001 vs distal) of 5 preparations for each group.

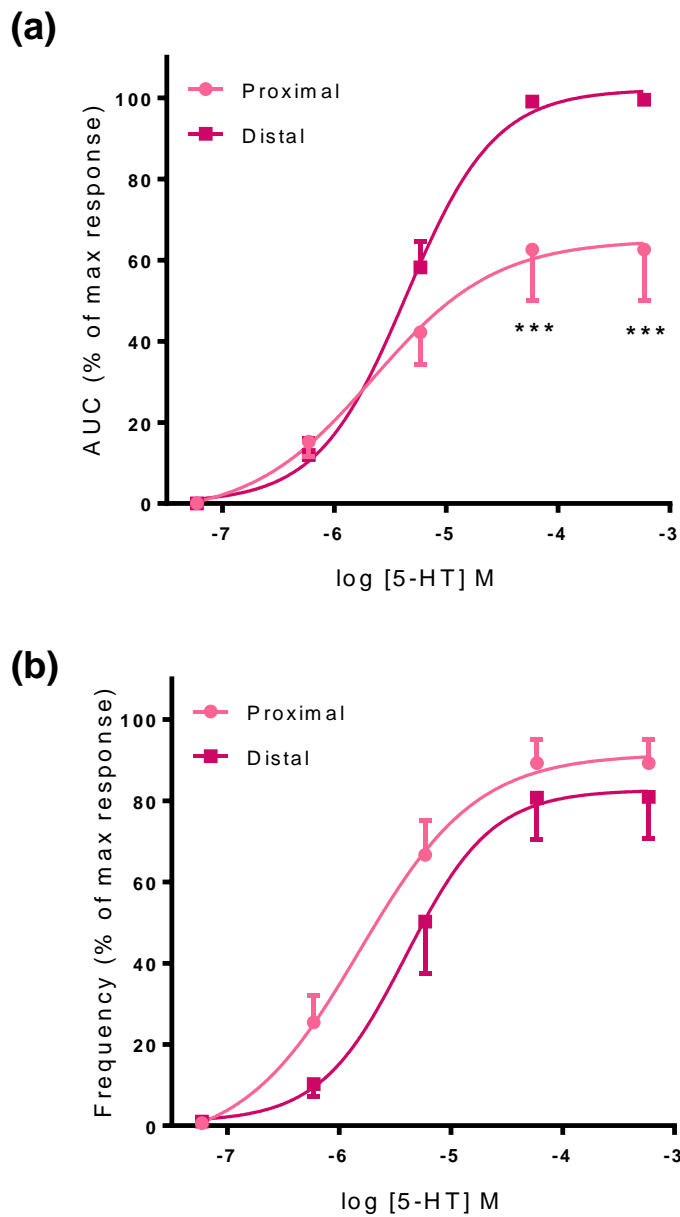
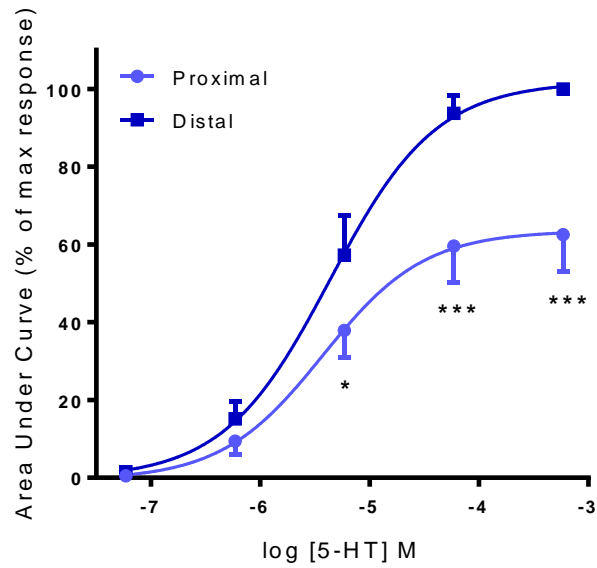


Figure 2.14 Concentration-response curves for 5-HT in the proximal and distal ureter from young pigs. Responses are expressed as percentage of maximal AUC (a) and frequency (b). Results are presented as mean \pm SEM (* p <0.05, ** p <0.01, *** p <0.001 vs distal) of 6 preparations for each group.

(a)



(b)

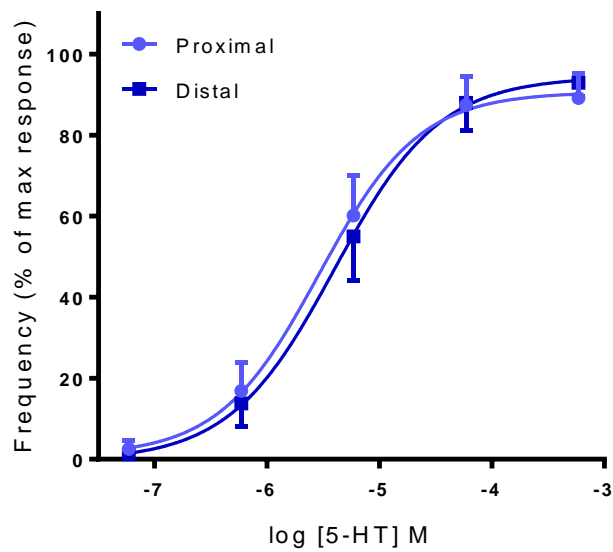


Figure 2.15 Concentration-response curves for 5-HT in the proximal and distal ureter from old pigs. Responses are expressed as percentage of maximal AUC (a) and frequency (b). Results are presented as mean \pm SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs distal) of 6 preparations for each group.

Table 2.6 Mean pEC₅₀ (\pm SEM) values for phenylephrine and 5-HT (AUC) in proximal and distal segments of pig ureter.

Agonist	Group	Segment	n	pEC ₅₀
Phenylephrine	Young	Proximal	6	5.01 \pm 0.61
		Distal	6	4.90 \pm 0.20
	Old	Proximal	5	5.15 \pm 0.12
		Distal	5	5.14 \pm 0.11
5-HT	Young	Proximal	6	5.67 \pm 0.32
		Distal	6	5.39 \pm 0.27
	Old	Proximal	6	5.61 \pm 0.36
		Distal	6	5.43 \pm 0.59

Discussion

The results indicate that there are differences in contractile responses between the proximal and distal ureter in specific age groups with specific agonists. A previous study in the isolated human ureter showed that maximum contraction to phenylephrine was significantly greater in the distal ureter in comparison to the proximal ureter (Sasaki et al., 2011). In a receptor-binding study, it was also shown that α_1 -adrenoceptor density was significantly greater in the distal human ureter than in the proximal ureter, which may explain why ureteral stones are more frequently lodged in the distal ureter (Sigala et al., 2005). A novel finding from our study shows that the larger phenylephrine-induced contractions in the pig isolated distal ureter compared to proximal ureter only occurs in older animals. Previous studies have not examined animals from different age groups. This result together with our previous finding where older animals

had increased maximal response to α_1 -adrenoceptor stimulation, could indicate that the adrenergic system plays an increased role in older animals in comparison to the younger animals, in the distal region of the ureter. This may be linked with increased reports of ureteral stones with age and the efficacy of α_1 -adrenoceptor antagonist, tamsulosin in increasing stone expulsion rate.

It was also observed that the number of ureteral strips from the proximal region that developed spontaneous contractions before addition of agonists was greater in comparison to strips from the distal ureter. This finding was as expected, due to the proximal ureter being located closer to the atypical smooth muscle cells located at the renal pelvis that initiate the peristaltic wave down the ureteral tube.

It is established that 5-HT induces ureteral contractions in isolated ureter preparations from various species including human (Hertle and Nawrath, 1986), pig (Hernandez et al., 2003) and dog (Dodel et al., 1996). However, this is the first study to compare contractile responses to 5-HT between the proximal and distal regions of the ureter. Isolated distal ureter from both animal age groups showed enhanced 5-HT-induced contractile responses in comparison to the proximal ureter, although frequency was similar. The difference in maximum AUC between the two regions was similar in both age groups. This indicates that 5-HT plays a greater role in contractility of the distal region of the ureter, independent of age.

In the isolated rat ureter, it has been reported that distal segments generate greater luminal pressures than proximal segments in response to acetylcholine (Rasidovic et al., 2010). Compared with distal segments, proximal segments were associated with a greater frequency of contraction and a lower magnitude of contraction (Rasidovic et al., 2010). Our findings partly support this, where maximum frequency of phasic contractions to phenylephrine of the isolated proximal ureter was greater

that distal segments in the younger animals. Additionally, isolated distal ureteral strips were able to achieve greater maximum AUC contractile responses in most cases which are indicative of increased magnitude of contractile responses.

As the distal ureter produced greater contractile responses, and it is also where most ureteral stones are lodged, we utilised the distal ureter in all subsequent experiments.

2.7 Effect of Indomethacin on Contractile Responses in the Ureter

Introduction

NSAIDs including indomethacin, ketorolac, and ibuprofen have in some clinical trials been shown to be effective for relief of pain during treatment of renal colic. In addition, many studies have suggested that prostanoids including prostaglandins, thromboxanes and prostacyclins are involved in contractile responses mediated by the ureter (Al-Ugaily et al., 1986, Mastrangelo et al., 2000, Davidson and Lang, 2000). The synthesis of prostanoids from arachidonate involves cyclo-oxygenase (COX), which exists in two isoforms: COX-1 and COX-2. Both isoforms have been identified in the ovine ureteral smooth muscle and urothelium by radioimmunoassay (Ali et al., 1998). COX-1 is expressed in most tissues and is responsible for most of the physiological prostaglandin synthesis and COX-2 is expressed at low levels in most cells but can be up-regulated locally by an inflammatory stimulus. Although this is the general understanding of these two different isoforms, the delineation of their individual roles is not as clear cut (Vane and Botting, 1995).

It is believed that NSAIDs relieve ureteral colic through their anti-diuretic and analgesic effects. However, it is likely that NSAIDs may have further therapeutic effects by inhibiting smooth muscle contractions in the ureter. Several studies have reported that there is a decrease in spontaneous, electrically-evoked and agonist-induced contractions in the ureter in various species in the presence COX inhibitors (Al-Ugaily et al., 1986, Mastrangelo et al., 2000, Davidson and Lang, 2000, Lee et al., 2010). However, the influence of age on the effect of COX inhibitor in the ureters has not been investigated. In this study, the aim was to determine the role

of COX in phenylephrine and 5-HT-induced contractile responses of the isolated pig distal ureter with age.

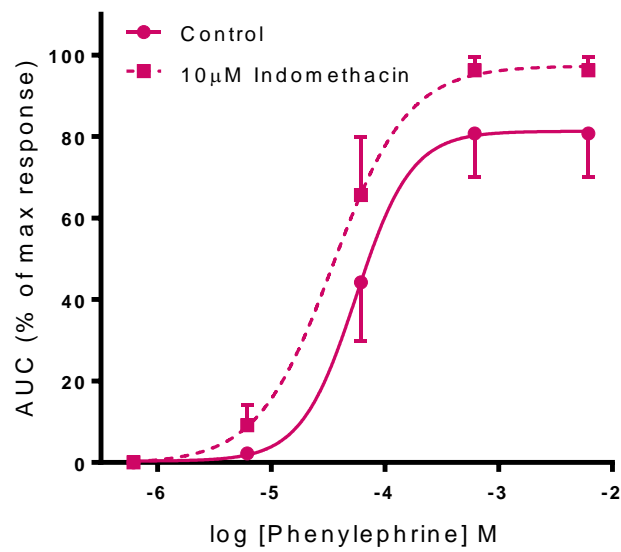
Methods and Materials

Tissue preparation and *in vitro* functional studies were performed as stated in Section 2.1. The agonists used were phenylephrine (7.48×10^{-8} – 7.48×10^{-4} M) and 5-HT (5.88×10^{-8} – 5.88×10^{-4} M). Contractile responses to these agonists were compared in the presence and absence of 10 μ M indomethacin which was introduced to the tissues before the first agonist concentration was added and allowed to incubate for 30 minutes. Contractions to agonists were expressed as maximum contractile responses in AUC and frequency of phasic contractions developed.

Results

A baseline of $1.23\text{g} \pm 0.15\text{g}$ (n=48) was achieved in tissues during the equilibration period and spontaneous contractions developed in 10 of 48 (21%) ureteral tissue strips. Indomethacin had no effect on potency (pEC_{50}) of either agonist in the isolated ureteral strips from both age groups (Table 2.7). Maximum AUC and frequency responses to phenylephrine were similar in the presence and absence of indomethacin (10 μ M) in ureteral tissues from both age groups (Figure 2.16 and 2.17). Maximum contractility to 5-HT stimulation expressed as AUC was decreased in the presence of indomethacin ($p < 0.05$, paired *t*-test, n=6, Figure 2.18a) in the younger animals but not in the older animals (Figure 2.19a). The maximum frequency of phasic contractions in response to 5-HT was unaffected by indomethacin in both age groups (Figure 2.18b and 2.19b).

(a)



(b)

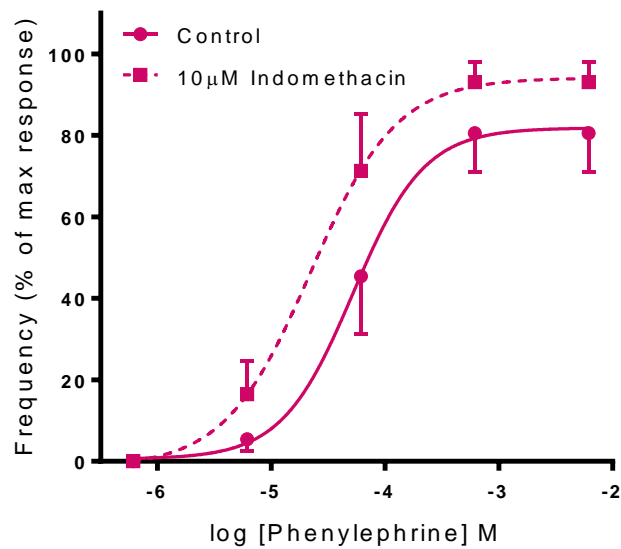


Figure 2.16 Concentration-response curves for phenylephrine in the distal ureter from young pigs in the absence and presence of indomethacin (10µM). Responses are expressed as percentage of maximal AUC (a) and frequency (b). Results are presented as mean \pm SEM of 6 preparations for each group.

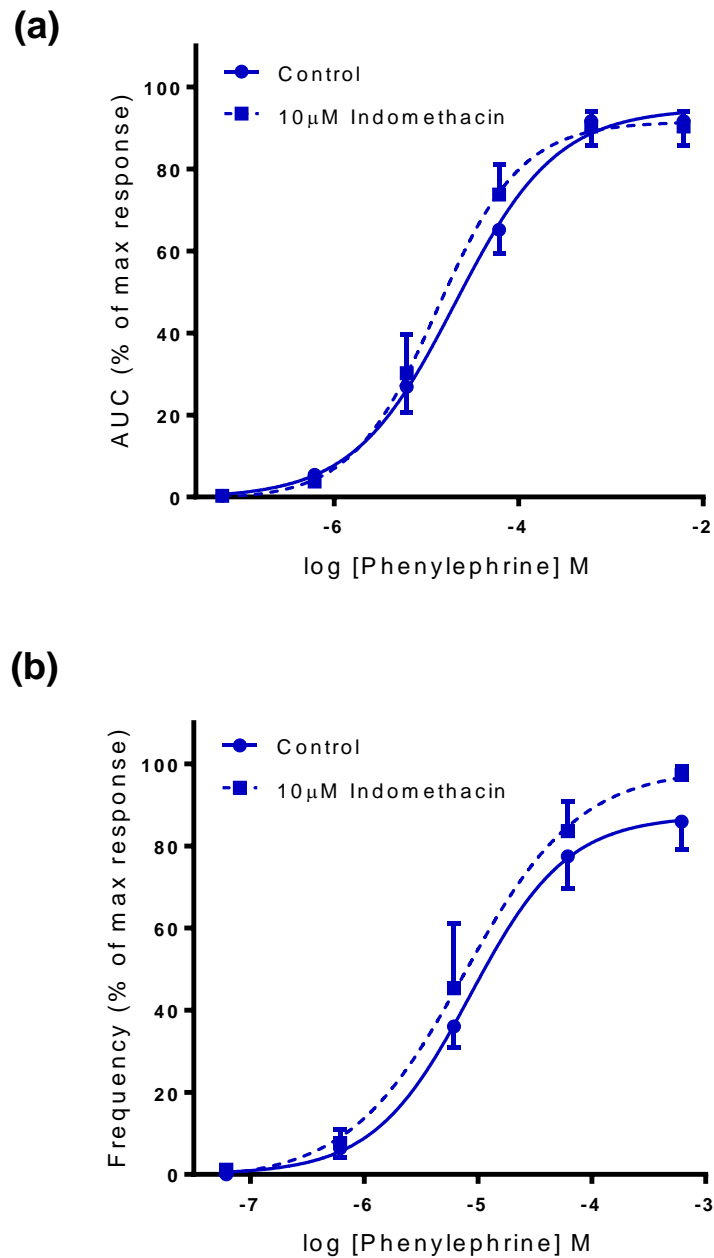


Figure 2.17 Concentration-response curves for phenylephrine in the distal ureter from old pigs in the absence and presence of indomethacin (10 μ M).

Responses are expressed as percentage of maximal AUC (a) and frequency (b). Results are presented as mean \pm SEM of 6 preparations for each group.

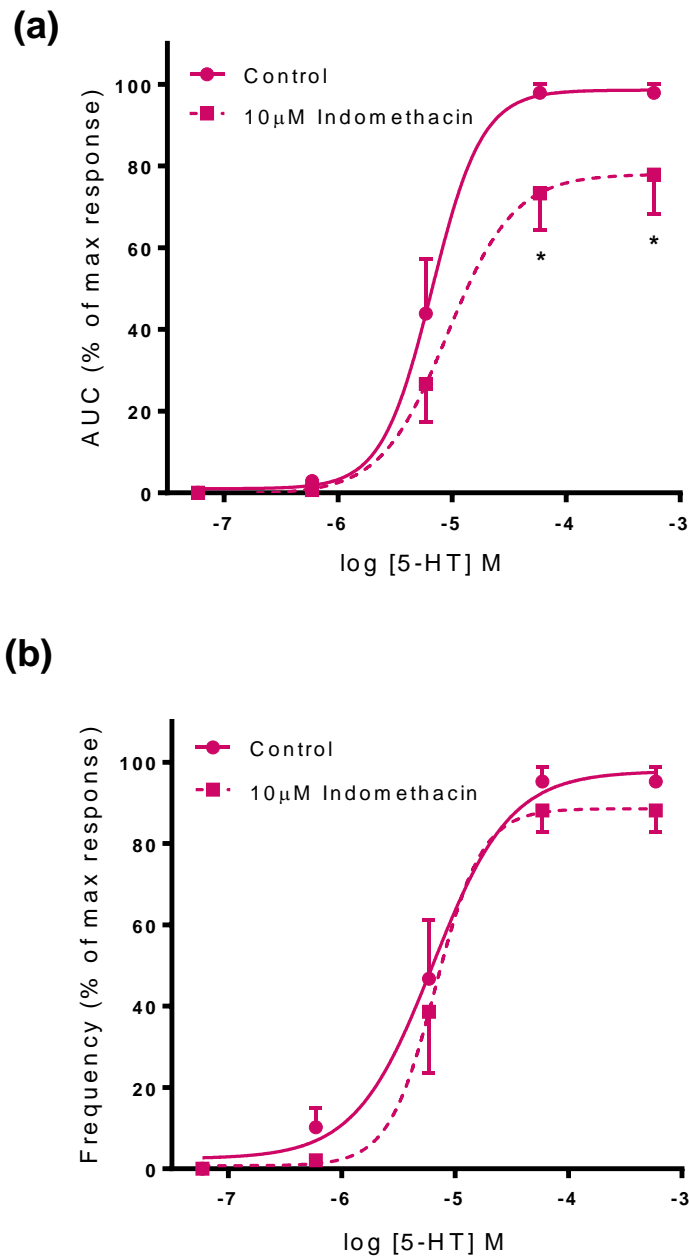
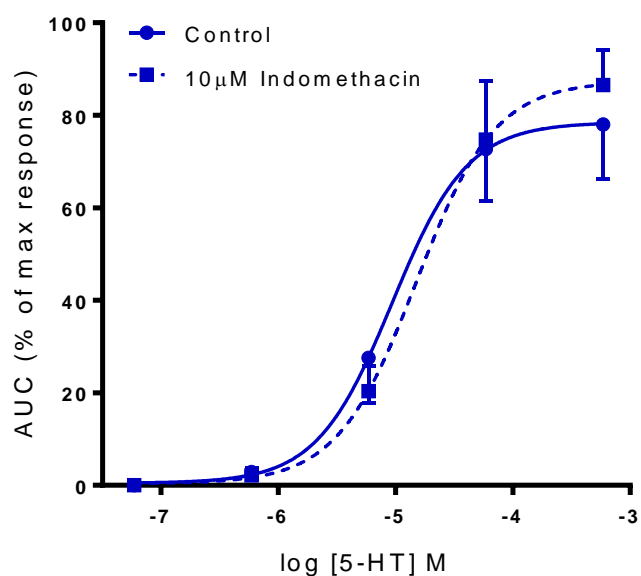


Figure 2.18 Concentration-response curves for 5-HT in the distal ureter from young pigs in the absence and presence of indomethacin (10 μM). Responses are expressed as percentage of maximal AUC (a) and frequency (b). Results are presented as mean \pm SEM of 6 preparations for each group.

(a)



(b)

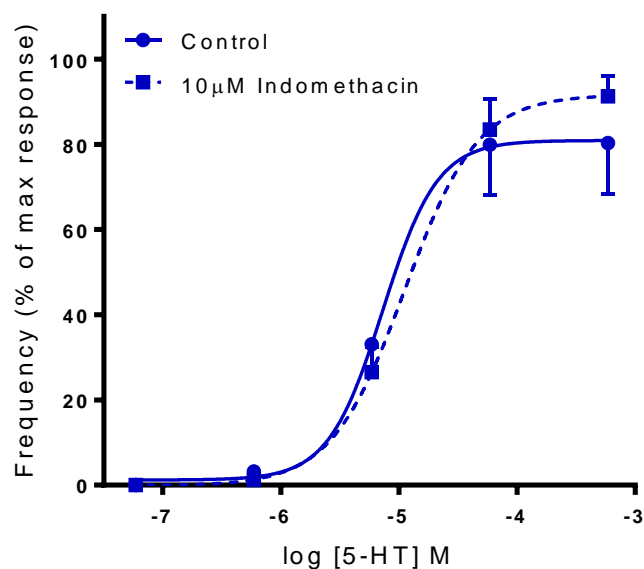


Figure 2.19 Concentration-response curves for 5-HT in the distal ureter from old pigs in the absence and presence of indomethacin (10 μ M). Responses are expressed as percentage of maximal AUC (a) and frequency (b). Results are presented as mean \pm SEM of 6 preparations for each group.

Table 2.7 Mean pEC₅₀ (\pm SEM) values (AUC) for phenylephrine and 5-HT in the pig distal ureter in the presence and absence of 10 μ M indomethacin.

Agonist	Group		n	pEC ₅₀
Phenylephrine	Young	Control	6	4.25 \pm 0.15
		10 μ M indomethacin	6	4.46 \pm 0.15
	Old	Control	6	4.69 \pm 0.14
		10 μ M indomethacin	6	4.89 \pm 0.13
5-HT	Young	Control	6	5.18 \pm 0.09
		10 μ M indomethacin	6	5.04 \pm 0.18
	Old	Control	6	5.03 \pm 0.22
		10 μ M indomethacin	6	4.83 \pm 0.18

Discussion

To investigate the role of COX in responses of the isolated ureter to phenylephrine and 5-HT, the non-selective COX-1 and COX-2 inhibitor indomethacin (10 μ M), was used. Previous studies of prostaglandins in the ureter have generally shown that COX inhibitors have a depressant effect on ureteral contractility. In the isolated human proximal ureter, NS-398, a selective COX-2 inhibitor induced dose-dependent relaxation of tonic and phasic contractions to KCl and Bay K 8644 (calcium channel agonist), whereas indomethacin and SC-650 (COX-1 inhibitor) had no effect (Lee et al., 2010). Diclofenac and NS-398 had similar depressant effects on pig ureteral contractions induced by various agonists including noradrenaline, 5-HT and neurokinin A (Mastrangelo et al., 2000).

Although the influence of age on the effects of prostaglandins have not been studied in the ureter, one previous study showed that both diclofenac and NS-398 caused a reduction in contractions to noradrenaline and 5-HT in isolated ureteral strips from young pigs (3 – 6 months) (Mastrangelo et al., 2000). Interestingly, this depressant effect of COX inhibitors was also observed in the present study for 5-HT-stimulated contractile responses in younger animals, but there was no effect of indomethacin on 5-HT-stimulated contractile responses in tissues from older animals. While that same study showed that response to norepinephrine was also attenuated by indomethacin (Mastrangelo et al., 2000), our result indicated otherwise. Outside of the urinary tract, a recent study on rat renal and femoral veins showed that indomethacin caused a statistically lower suppression of phenylephrine-induced responses in comparison to responses to noradrenaline (de Souza Rossignoli et al., 2015). It was suggested that this difference may be due to noradrenaline acting simultaneously on both α_1 - and α_2 -adrenoceptors and that local modulatory mechanisms such as prostanoid release affect contractile responses with a synergism between the α_1 -adrenoceptor subtypes (de Souza Rossignoli et al., 2015). However, because phenylephrine stimulates only α_1 -adrenoceptor, it could possibly explain our observation where phenylephrine-induced contractile responses were not affected by indomethacin as was observed in 5-HT-stimulated contractile responses. Therefore, it is suggested that in the younger animals, indomethacin plays a role in the inhibition of contractile responses induced by 5-HT.

While the effects of ageing on the activity of COX in the urinary tract have not been clearly elucidated, studies have suggested that in pig pulmonary artery indomethacin caused a rightward shift of the phenylephrine concentration-response curve in young (5 weeks old) piglets but not in older (26 weeks old) pigs (Gustin et al., 1993). However, in a separate study, neither cyclo-oxygenase nor phospholipase A₂ activity was altered as a function of age in guinea-pig whole blood (Spaethe et al., 1992).

There was also no influence of age on cyclo-oxygenase activity in rat aortic smooth muscle cells (Chang et al., 1980).

We conclude that the impact of ageing on the effect of COX inhibitors on contractile responses to phenylephrine and 5-HT of smooth muscle cells might vary between different physiological systems. In the ureter, they appear to have an inhibitory effect in younger animals but no effect on older animals. This is an interesting finding as it proposes that prostanoids might only play a role in modulation of ureteral motility in younger animals, but not in the older animals.

2.8 Effect of L-N^G-Nitroarginine (L-NNA) on Responses in the Ureter

Introduction

Nitric oxide (NO) is a gaseous molecule mediating relaxation or inhibition in many biological processes, acting as a second messenger and neurotransmitter. The enzyme NO synthase (NOS) is responsible for NO synthesis from L-arginine and this only occurs in the presence of co-factors, such as nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase (Fernandes and Hernandez, 2016). NO induces relaxation in smooth muscle cells of the lower urinary tract via soluble guanylate cyclase (sGC). The binding of NO with sGC causes a change in the enzyme conformation which increases the catalytic rate of conversion of guanosine triphosphate to the second messenger, cyclic guanosine monophosphate (cGMP). This has been shown to induce relaxation via the opening of potassium channels in the human ureter (Miyaoka et al., 2014).

NO has been observed in nerve fibres in the lower urinary tract and plays a prominent role in non-adrenergic, non-cholinergic neurotransmission in efferent pathways to the lower urinary tract (Andersson and Persson, 1995). Additionally, it has been demonstrated that NOS-immunoreactive nerves have an inhibitory effect on trigonal, bladder neck and urethral smooth muscle tone (Andersson and Persson, 1995). There is evidence suggesting that in the ureter, NO is released from the urothelium and is a major inhibitory transmitter involved in ureteral peristalsis, urinary flow resistance and local blood flow (Iselin et al., 1997). It has also been found that NO donors reduce the frequency of spontaneous rhythmic contractions in the renal pelvis and proximal ureter and could be a

potential strategy for treatment of ureteral calculi (Mastrangelo et al., 2003).

In the pig intravesical ureter, addition of L-NNA, abolished electrically induced relaxation which was significantly reversed by addition of L-arginine (Hernandez et al., 1995). This effect by L-NNA was also observed for electrically-induced responses of sheep ureter (Levent and Buyukafsar, 2004). It was therefore proposed that NO synthase is present in the nerve fibres and they seem to be involved in the mediation of inhibitory neurotransmission. However, in the sheep urinary tract, NO-mediated relaxation of the ureter could not be detected (Garcia-Pascual et al., 1996). Studies involving NO in the ureter have mostly focused on electrically induced contractile responses (Hernandez et al., 1995, Fernandes and Hernandez, 2016). In addition to this, the effect NO has on contractile activity in different age groups has also not previously been investigated. We aimed to investigate the effects of L-NNA on phenylephrine and 5-HT-induced contractile activity in old and young pigs.

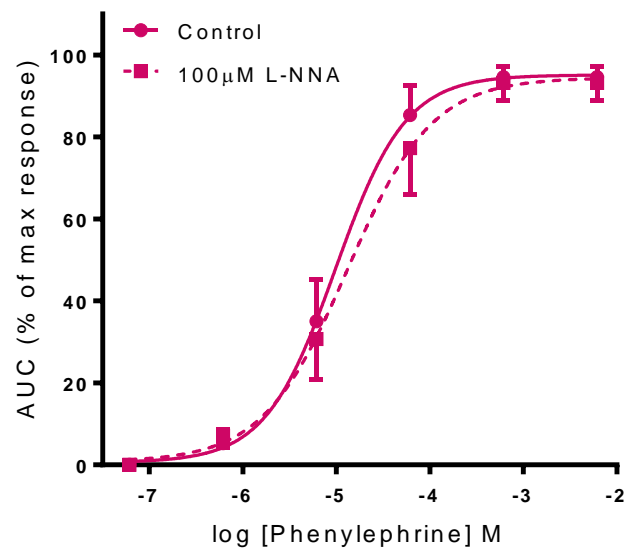
Methods and Materials

Tissue preparation and *in vitro* functional studies were performed as stated in Section 2.1. The agonists used in this study were phenylephrine (7.48×10^{-8} – 7.48×10^{-4} M) and 5-HT (5.88×10^{-8} – 5.88×10^{-4} M). Contractile responses to these agonists were compared in the presence and absence of L-NNA (100 μ M) which was incubated for 30 minutes before addition of agonist. Contractions to agonists were expressed as percentage of maximal response in AUC and frequency of phasic contractions developed.

Results

Spontaneous contractions developed in 8 of 48 (17%) isolated distal ureteral strips during equilibration to a passive tension of $1.15\text{g} \pm 0.10\text{g}$ ($n=48$). L-NNA ($100\mu\text{M}$) had no effect on potency (pEC_{50}) of both agonists in ureteral strips from both age groups (Table 2.8). Additionally, maximum AUC and frequency responses to phenylephrine and 5-HT were unaffected in the presence L-NNA ($100\mu\text{M}$) in both age groups (Figure 2.13 and 2.14).

(a)



(b)

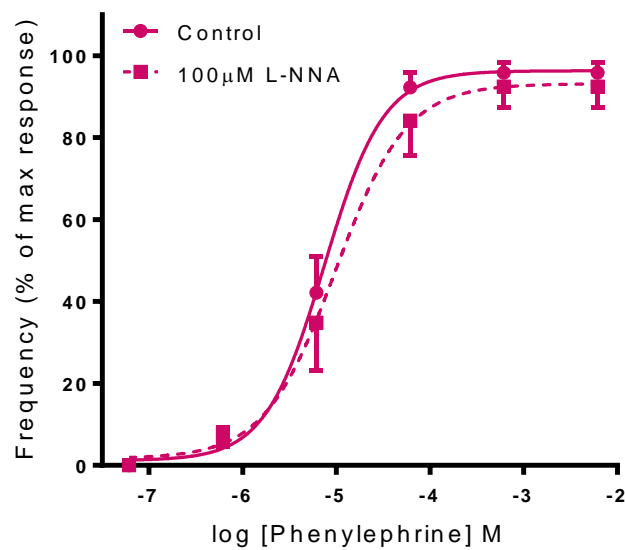


Figure 2.20 Concentration-response curves for phenylephrine in the distal ureter from young pigs in the absence and presence of L-NNA (100 μ M).

Responses are expressed as percentage of maximum AUC (a) and frequency (b). Results are presented as mean \pm SEM of 6 preparations for each group.

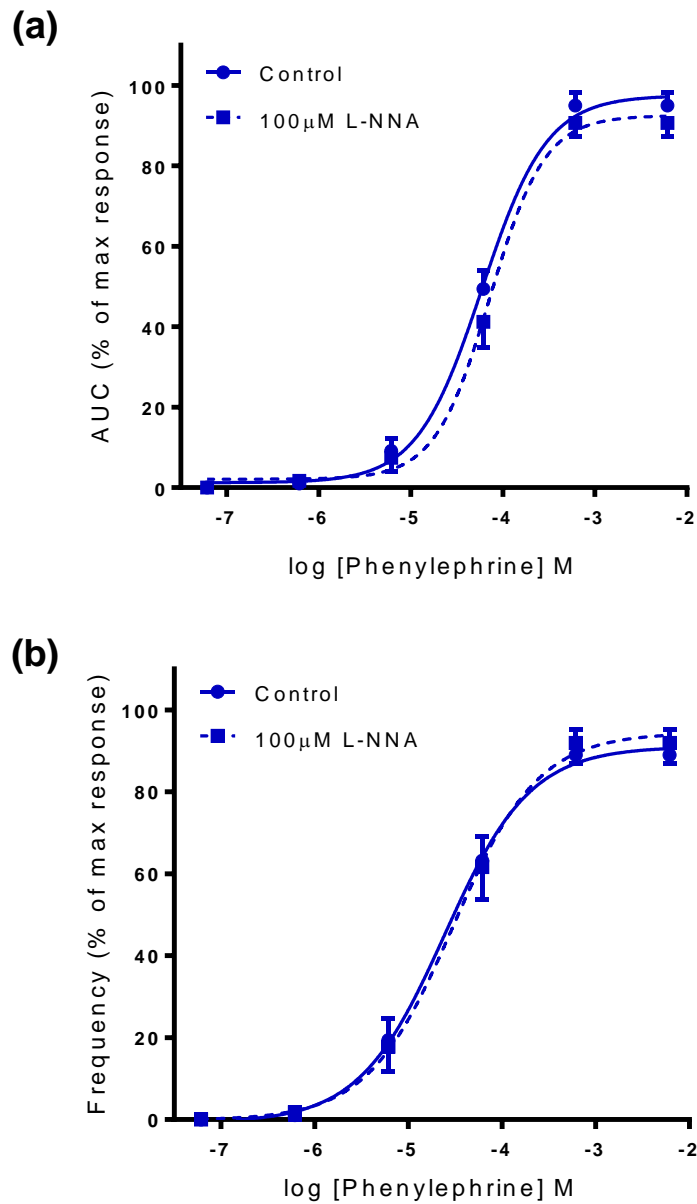
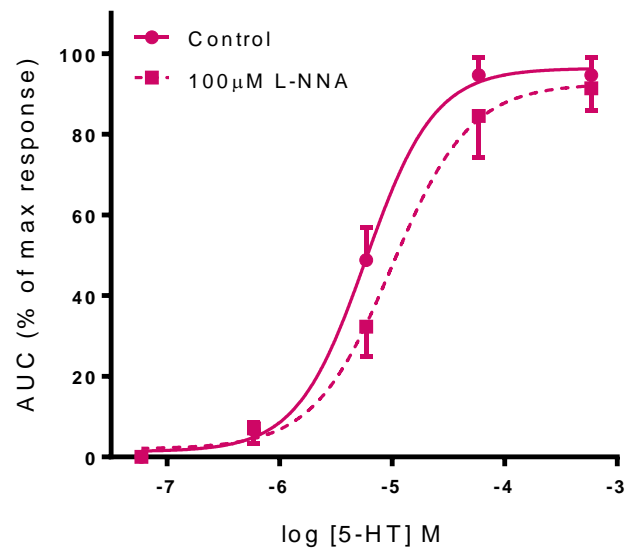


Figure 2.21 Concentration-response curves for phenylephrine in the distal ureter from old pigs in the absence and presence of L-NNA (100 μ M). Responses are expressed as percentage of maximum AUC (a) and frequency (b). Results are presented as mean \pm SEM of 6 preparations for each group.

(a)



(b)

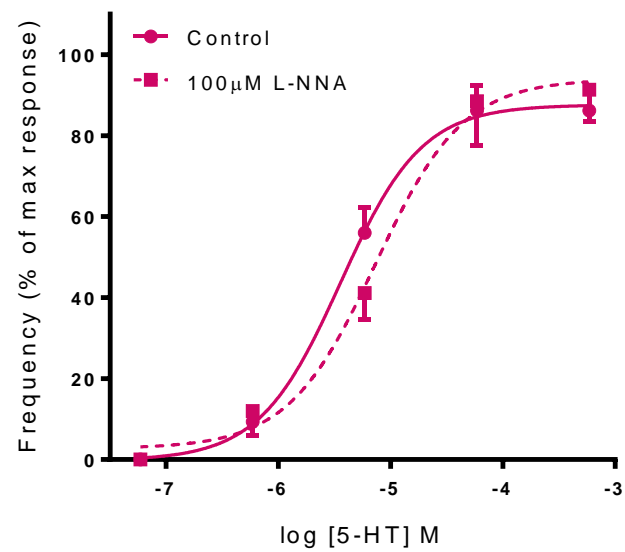
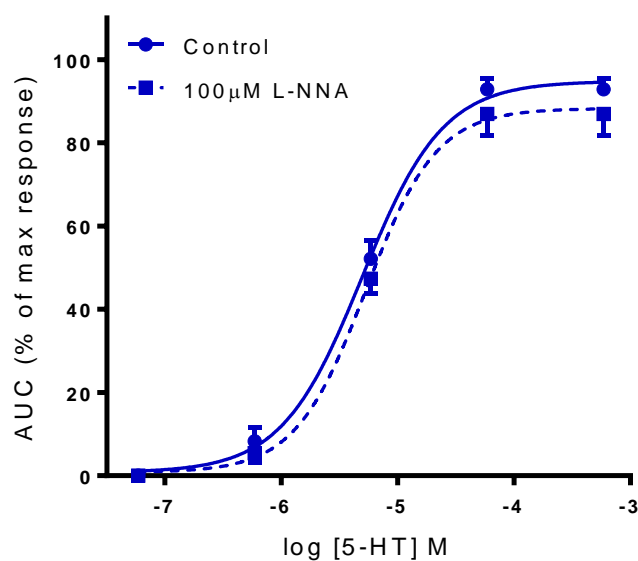


Figure 2.22 Concentration-response curves for 5-HT in the distal ureter from young pigs in the absence and presence of L-NNA (100 μ M). Responses are expressed as percentage of maximum AUC (a) and frequency (b). Results are presented as mean \pm SEM of 6 preparations for each group.

(a)



(b)

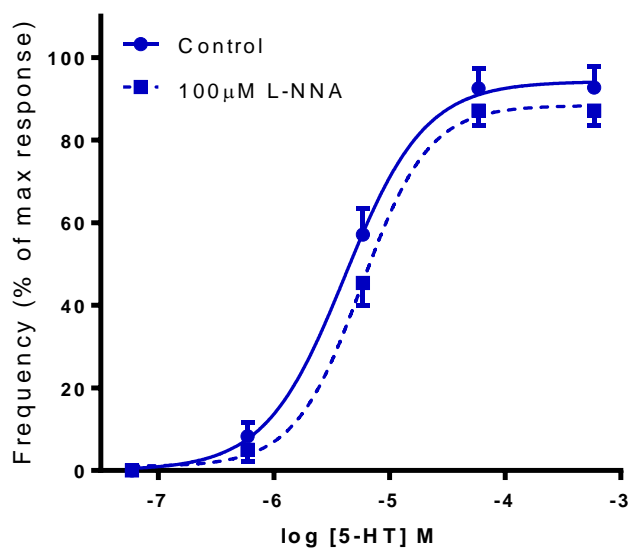


Figure 2.23 Concentration-response curves for 5-HT in the distal ureter from old pigs in the absence and presence of L-NNA (100 μ M). Responses are expressed as percentage of maximum AUC (a) and frequency (b). Results are presented as mean \pm SEM of 6 preparations for each group.

Table 2.8 Mean pEC₅₀ values (\pm SEM) for phenylephrine and 5-HT in the pig distal ureter in the presence and absence of L-NNA (100 μ M). Results are expressed as mean \pm SEM.

Agonist	Group		n	pEC ₅₀
Phenylephrine	Young	Control	6	4.68 \pm 0.11
		100 μ M L-NNA	6	4.64 \pm 0.28
	Old	Control	6	4.23 \pm 0.05
		100 μ M L-NNA	6	4.14 \pm 0.06
5-HT	Young	Control	6	5.24 \pm 0.07
		100 μ M L-NNA	6	5.00 \pm 0.15
	Old	Control	6	5.33 \pm 0.06
		100 μ M L-NNA	6	5.17 \pm 0.08

Discussion

In this study, we examined the effect of the NOS inhibitor L-NNA, on phenylephrine and 5-HT-induced contractile responses of ureter from two age groups. We demonstrated that NO was not involved in responses of the pig distal ureter to phenylephrine and 5-HT.

Many studies have suggested that NO plays an important inhibitory effect on electrical field-stimulated contractions. However, our results indicate that there is no α_1 -adrenoceptor-stimulated or 5-HT receptor-stimulated release of NO in these tissues. Evidence showing localisation of NO synthase at the porcine intravesical ureter suggests that NO is a non-adrenergic, non-cholinergic transmitter involved in the inhibitory system (Hernandez et al., 1995). However, in this same study, it was shown that

NANC responses were only observed in the presence of guanethidine and atropine, which inhibit adrenergic and cholinergic transmission respectively (Hernandez et al., 1995). This could explain the lack of effect of L-NNA in the present study since we did not inhibit adrenergic or cholinergic transmission.

One study has shown that exogenous NO (SIN-1) temporarily abolishes contractions of the isolated pig distal ureter to 5-HT (Iselin et al., 1996). However, at maximum concentrations of SIN-1, 5-HT responses were attenuated by less than 50% (Iselin et al., 1996). It is possible that in the presence of NO donors, ureteral contractile responses to phenylephrine will also be attenuated, as this effect was also shown for isolated pig bladder tissue responses to muscarinic receptor stimulation (Moro et al., 2012). It was shown in the rat, that urothelial muscarinic receptors induce NO release but only after the induction of inducible NO synthase in conditions such as bladder inflammation (Giglio et al., 2005).

A study examining the distribution of NOS-immunoreactive nerves in the rat distal ureter showed that aged animals had a lower density of nerves compared to the younger animals (Mohammed and Santer, 2001). In addition, it was also shown that NOS-immunoreactive nerves and NADPH-diaphorase in the extramural ganglion complex that provides autonomic innervation to the distal ureter, ureterovesical junction and bladder trigone are also downregulated with age (Mohammed and Santer, 2001, Bergman et al., 1999). This raises the possibility that the relaxation function of NO and the transmission of sensory information may be compromised with increasing age in the ureter and bladder.

In conclusion, several studies in the literature suggest that the responses of the ureter are depressed by NO. The precise mechanism is not clearly understood, however, since L-NNA does not affect phenylephrine or 5-HT

responses in the present study, it would appear neither α_1 -adrenoceptor nor 5-HT receptor stimulation causes NO release.

CHAPTER 3:
Characterization of the 5-HT receptor
subtype in the porcine ureter

3.1 Introduction

5-Hydroxytryptamine (5-HT) is a well-known mono-amine transmitter that not only plays significant roles in the central nervous system but also in the regulation of smooth muscle contractions in several systems including in the gastrointestinal, vascular and urinary systems (Nichols and Nichols, 2008). In mammals, 5-HT is synthesised biologically via two enzymatic steps: firstly, ring hydroxylation of the amino acid tryptophan by tryptophan hydroxylase, which is the rate-limiting step, and secondly, side chain decarboxylation by aromatic amino acid decarboxylase (Nichols and Nichols, 2008).

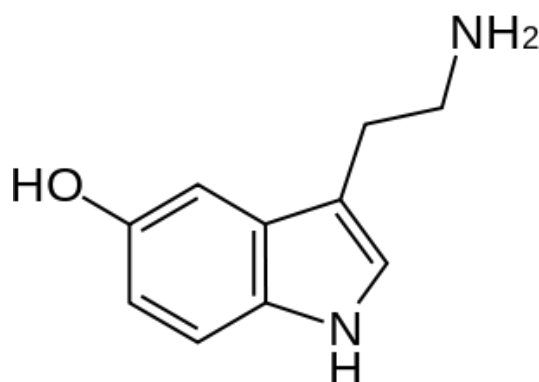


Figure 3.1 Structure of 5-HT (Nichols and Nichols, 2008)

The receptors for 5-HT are typically classified according to their primary signalling mechanism into four subtypes: the 5-HT₂ (A, B, C) receptors acting via phospholipase C activation, 5-HT₄, 5-HT₆, and 5-HT₇ receptors via activation of adenylyl cyclase, 5-HT₁ (A, B, D, E, F) and 5-HT₅ (A, B) via adenylyl cyclase inhibition and 5-HT₃, a ligand-gated ion channel (Nichols and Nichols, 2008).

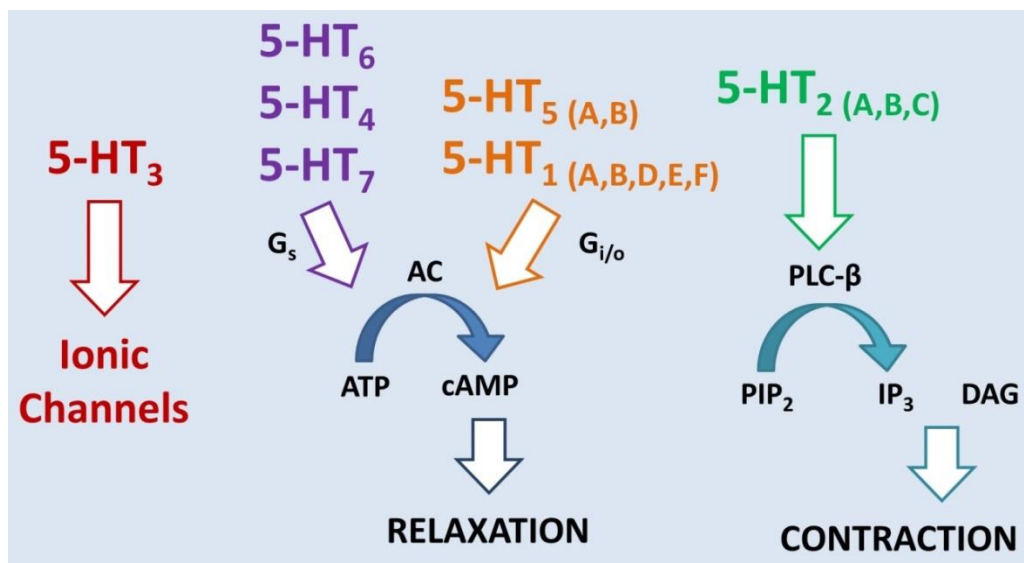


Figure 3.2 Diagrammatic representation of 5-HT receptor subtypes and their second messenger pathways.

5-HT is primarily found in platelets, the gastrointestinal tract and brain and is capable of stimulating smooth muscle contraction and/or relaxation in various tissues including the intestine, vasculature and urinary bladder (Klarskov and Horby-Petersen, 1986, Kim and Camilleri, 2000, Mohammad-Zadeh et al., 2008). 5-HT can have both pre- and post-junctional actions in the mammalian urinary tract, where it can have direct actions on smooth muscle cells or indirect effects on the autonomic intramural neurons (Hernandez et al., 2003). 5-HT can stimulate micturition in intact animals and has excitatory actions in human detrusor strips (Klarskov and Horby-Petersen, 1986, Corsi et al., 1991). In addition to this, there is also evidence that 5-HT can modulate peripheral sympathetic neurotransmission through inhibition of noradrenaline release via pre-junctional 5-HT₁-like receptors, which have now been characterized as a mixture of 5-HT_{1B}, 5-HT_{1D} and 5-HT₇ (Saxena et al., 1998). Although this is the primary mechanism, there is also evidence that

5-HT can enhance adrenergic neurotransmitter release via facilitatory pre-junctional 5-HT_{1A} receptors (Cohen et al., 1999).

While the mammalian urinary bladder and lower urinary tract have been shown to respond to 5-HT with contraction, in many studies in the human and pig (Todd and Mack, 1969, Ambache and Zar, 1970, Long and Nergardh, 1978), others have proposed the opposite effect; a relaxation (Klarskov and Horby-Petersen, 1986). 5-HT induces contraction of the detrusor smooth muscle cells by enhancement of cholinergic transmission through mechanisms involving neural 5-HT₃ receptors in the rabbit (Chen, 1990) and guinea-pig (Messori et al., 1995). Besides that, there are also reports suggesting the role of ketanserin-sensitive muscular 5-HT₂ receptors in 5-HT-evoked contractile response in human (Klarskov and Horby-Petersen, 1986) and dog (Cohen, 1990) urinary bladder. The presence of pre-junctional modulation by 5-HT in the lower urinary tract has also been demonstrated in numerous species, including the human (Tonini et al., 1994), pig (Sellers et al., 2000) and monkey (Waikar et al., 1994) bladder via 5-HT₄ receptors, whose activation results in potentiation of cholinergic contractile responses. Experimental studies in animals have demonstrated that spinal reflex circuits involved in voiding function exhibit a dense serotogenic innervation and multiple 5-HT receptors (Khaled and Elhilali, 2003). Expression of several subtypes of 5-HT receptor including 5-HT_{2A}, 5-HT_{2B}, 5-HT₄ and 5-HT₇ has been demonstrated in the rat urinary bladder; particularly, 5-HT₂ receptors, being involved in 5-HT induced contractile responses (Sakai et al., 2013).

In the ureter, findings have been less complex than in the bladder, where in all species examined thus far, 5-HT has been found to produce an excitatory action only and does not induce relaxation of smooth muscle cells. *In vivo* studies have also demonstrated that intravenous administration of 5-HT and 2,5-dimethoxy-4-iodoamphetamine (DOI), a 5-HT_{2A/2C} receptor agonist, increase ureteral contraction frequency of the pig

ureter in a concentration-dependent manner (Hauser et al., 2002). Generally, *in vitro* studies have demonstrated that 5-HT can increase both phasic and tonic contractions in isolated ureter from dog (Klarskov and Horby-Petersen, 1986), human (Gidener et al., 1999, Gidener et al., 1995) and pigs (Hernandez et al., 2003). In the pig intravesical ureter, it was suggested that the 5-HT_{2A} receptor is the subtype involved in mediating 5-HT-induced contractile tone (Hernandez et al., 2003). However, responses to 5-HT in the pig intravesical ureter were different to those observed in the pig distal ureter, since 5-HT produced only an increase in contractile tone in the intravesical ureter and did not trigger bursts of phasic activity (as reported in Chapter 2) (Hernandez et al., 2003). Thus, a complete pharmacological characterization of the 5-HT receptor subtype/s involved in 5-HT-stimulated responses in the distal ureter has not yet been accomplished.

Furthermore, the regulation of smooth muscle contraction by 5-HT and its receptors has been associated with disorders of the lower urinary tract, including the bladder. Normal bladder function requires proper coordination of detrusor relaxation during the filling phase and contraction during micturition. In the rat urinary bladder, it was shown that 5-HT-induced bladder contractile responses are enhanced approximately 3-fold after partial bladder outlet obstruction (Sakai et al., 2013, Michishita et al., 2015). Additionally, it has been suggested that 5-HT modulation of cholinergic responses could be altered in bladder dysfunction. An increased 5-HT₄ receptor-mediated response in detrusor smooth muscle strips from patients with hyper-reflexic bladders has been demonstrated (Sellers et al., 2000), highlighting 5-HT₄ receptor antagonists as a possible therapeutic treatment for bladder voiding defects associated with hypercontractility. In addition to this, mRNA expression for 5-HT_{2A} and 5-HT_{2B} receptors in the detrusor muscle and subserosal layer of the bladder was also found to be up-regulated in bladder hyperactivity and also in

partial bladder outlet obstruction (Sakai et al., 2013, Michishita et al., 2015).

Similar to the bladder, ureteral peristalsis is also dependent on the normal functioning of ureteral smooth muscle contraction. In the rabbit distal ureter, induced partial or complete ureterovesical junction obstruction enhanced contractile responses to 5-HT, the latter producing twelve-fold greater augmentation than partial obstruction and signifying an apparent sensitization of the ureter to 5-HT in this disorder (Yalcin et al., 2013).

Ageing has been shown to increase the risk of ureteral calculus development (Hess, 2003). Whilst research on age-related changes in 5-HT contractile mechanism in the urinary tract is not readily available, there are observed changes in other organ systems involving smooth muscle contraction. In the gastrointestinal system, where 5-HT plays a vital role, immunohistochemical studies have indicated a decrease in 5-HT expressing enterochromaffin cells and attenuated serotogenic signalling, in 24-month old rats compared to 3- and 12-month old rats (Keating et al., 2015). Similarly, esophageal smooth muscle relaxation in response to 5-HT was also demonstrated to be significantly reduced with ageing in isolated tissue strips from rats (Tugay et al., 2003). Additionally, the inhibitory effect of 5-HT via 5-HT₂ receptors in the rat vas deferens was also found to gradually decrease with increasing age of the rats (Moritoki et al., 1986). These reports suggest possible alterations of the 5-HT systems across the various physiological systems with ageing. However, in contrast, no alteration with age was observed in tracheal smooth muscle contractile response to 5-HT in guinea pigs, although a possible explanation for this is the small range of age (3-4weeks vs 4-8weeks old) that was examined (Cox and Cohen, 1994).

In our preliminary data (Chapter 2), it was observed that contractile responses of isolated distal ureter to 5-HT in older animals were

depressed in comparison to tissues from younger animals. In addition, it was found that 5-HT exerts a major contractile effect in the ureteral wall, as it was able to produce approximately 3-fold greater contractions than muscarinic receptor stimulation (Chapter 2). Therefore, this leads to the possibility of 5-HT contractile mechanisms being a target for future drug development, and a potential use in medical expulsive therapy for treatment of ureteral stones. While it has been well established by numerous studies that 5-HT can produce contraction of ureteral smooth muscle, the receptor subtype mediating these contractions has not been distinctly characterized. Therefore, the aim of this study was to perform a full pharmacological analysis to functionally characterize the 5-HT receptor subtype/s mediating 5-HT-induced contractile responses in porcine isolated distal ureter. Furthermore, our study also investigated the effect of age on these responses, using tissues from two age groups: young (3 months) and old (24 months) pigs.

3.2 Methods and Materials

Tissue preparation and *in vitro* functional studies were performed as described in Chapter 2. For the first part of the study, isolated ureteral tissue strips were paired and were exposed to either increasing doses of 5-HT ($5.88 \times 10^{-8} - 5.88 \times 10^{-4}$ M) or the 5-HT₂ agonist α -methyl-5-HT ($6.57 \times 10^{-8} - 1.97 \times 10^{-4}$ M) and responses compared. In the second part of the study, isolated tissues were paired, with one strip used as a control strip, while the other was incubated for 20 minutes with one of the various 5-HT receptor subtype antagonists listed in Table 3.1, before being subjected to increasing concentrations of 5-HT. In addition to this, antagonist experiments with ketanserin (10 – 100nM) were repeated using the agonist α -methyl-5-HT. Concentrations that were chosen for the various 5-HT antagonists are based on literature values that have been shown to block the respective 5-HT receptor subtypes as shown in Table 3.1.

Paired student's *t*-tests were performed on studies with two groups while two-way ANOVA was used for studies involving more than two groups.

Table 3.1 Antagonist and agonist affinities (pKd) at different 5-HT receptor subtypes.

Antagonist/ Agonist	1A	1B	1D	1E	1F	2A	2B	2C	3	4	5A	5B	6	7
Methiothepin	7.7 ¹	8.1 ¹	7.7 ¹			9.0 ¹					7.0 ¹	6.6 ¹	8.7 ¹	8.4 ¹
Ketanserin	<6.0 ¹	5.3 ¹	7.2 ¹	<6.0 ¹	<6.0 ¹	8.7 ²	5.8 ²	6.9 ²			4.8 ¹	4.8 ¹	<5.0 ¹	6.0 ¹
RS-102221								8.7 ⁴						
Ondansetron									8.6 ¹	<4 ¹				
GR-113808	<5.0 ¹	<5.0 ¹	<5.0 ¹	<5.0 ¹	<5.0 ¹	<5.0 ¹		<5.0 ¹	6.0 ¹	9.7 ¹				
SB 699551		<5.5 ⁵	<5.5 ⁵			<6.0 ⁵		<6.0 ⁵			8.3 ⁵			<5.5 ⁵
SB 399885													9.0 ⁶	
SB 269970	<5.0 ¹	6.0 ¹	5.8 ¹	<5.2 ¹	<5.5 ¹	<5.0 ¹	5.9 ¹	<5.0 ¹		5.9 ¹	7.2 ¹		5.2 ¹	8.9 ¹
α -methyl-5-HT	6.0 ¹					8.4 ³	6.1 ³	7.3 ³		5.4 ¹				

¹ (Hoyer et al., 1994) ² (Bonhaus et al., 1995) ³ (Baxter et al., 1995) ⁴ (Bonhaus et al., 1997) ⁵ (Thomas et al., 2006) ⁶ (Foley et al., 2008)

3.3 Results

Porcine ureteral strips were allowed to equilibrate to a mean passive tension of $1.10\text{g} \pm 0.33\text{g}$ ($n=276$). Spontaneous contractions developed during equilibration period in 41 of 276 isolated distal ureteral strips (15%). Current literature suggests that the most likely candidate that sets spontaneous contractions in the ureter are 'atypical' smooth muscle cells, which are found in the proximal ureter and not in the distal ureter (Lang and Klemm, 2005). Electrical and mechanical activities are initiated by these spontaneously active cells in the pelvi-calyceal region of the renal pelvis and conducted to distal regions in the ureter that are not normally active unless driven by pacemakers (Berridge, 2008). Therefore, it is suggested that the spontaneous contractions that developed in 15% of distal ureter preparations are due to pacemaker signals before dissection that are still present. To further validate this suggestion, these spontaneous contractions usually disappear at the end of the incubation period.

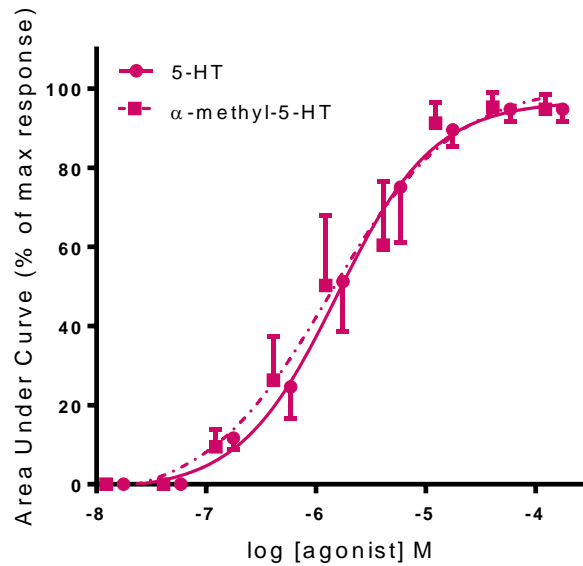
When subjected to increasing doses of 5-HT and the 5-HT₂ selective agonist α -methyl-5HT, all ureteral strips from both age groups developed bursts of phasic contractions. Increasing doses of 5-HT and α -methyl-5-HT also resulted in an increase in frequency of phasic activity. No significant differences were found between 5-HT and α -methyl-5-HT contractile responses in isolated distal ureter from either older or younger animals (Figure 3.3 and 3.4).

Ketanserin (10 – 100nM), a selective 5-HT_{2A} antagonist, produced a significant rightward shift of the 5-HT concentration-response curve (Figures 3.5a, 3.6a, 3.7a and 3.8a). In addition to this, maximal contractile responses to 5-HT expressed as AUC and frequency were depressed in the presence of ketanserin (Figures 3.5a, 3.6a, 3.7a and 3.8a). Max

responses could not always be obtained but a comparison of the slopes of the concentration-response curves in each group was performed and there was no significant difference in any data group ($p>0.05$), suggesting competitive antagonism. Using EC_{40} values for responses (expressed as AUC and frequency), Schild plots were developed for tissues from both age groups (Figures 3.5b, 3.6b, 3.7b and 3.8b). The slopes of the Schild plots were not significantly different from unity, again suggesting a competitive antagonism, except for the frequency of phasic contractions in tissues from older animals, where the slope was significantly steeper than unity (Fig 3.8b). pA_2 values were also estimated by extracting the x -intercept of Schild linear regressions. These values are stated in Table 3.2 below.

Contractile Responses to 5-HT and α -Methyl-5HT

(a)



(b)

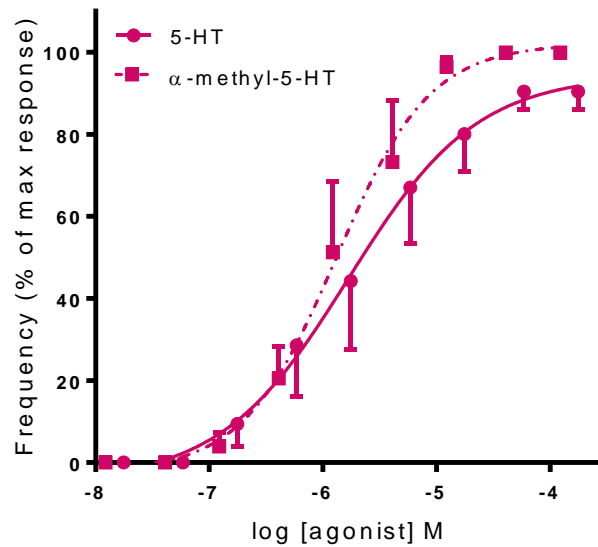
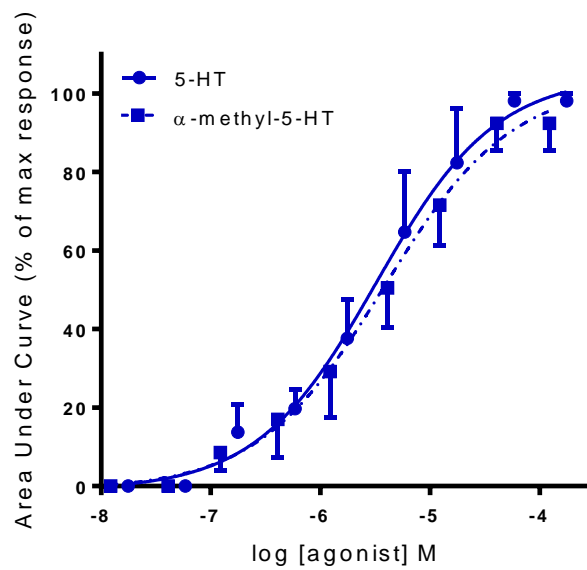


Figure 3.3 Concentration-response curves for **5-HT** and **α -methyl-5-HT** in the distal ureter from young pigs in isolated smooth muscle strips. Responses are expressed as percentage of maximal response for AUC (a) and frequency (b). Results are presented as mean \pm SEM of 4 preparations for each group.

(a)



(b)

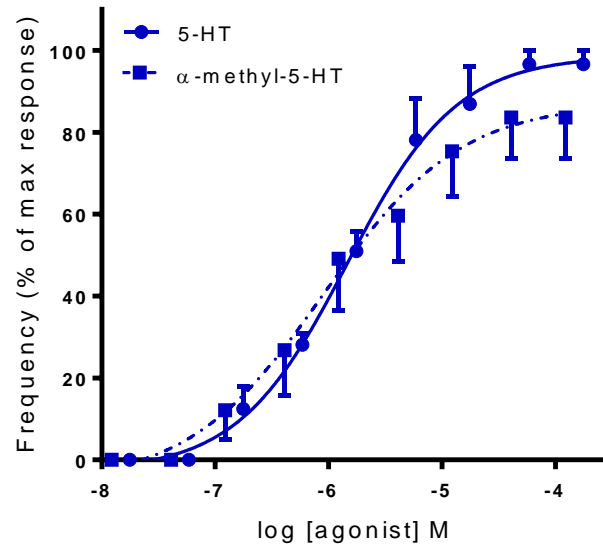


Figure 3.4 Concentration-response curves for 5-HT and α -methyl-5-HT in the distal ureter from old pigs in isolated smooth muscle strips. Responses are expressed as percentage of maximal response for AUC (a) and frequency (b). Results are presented as mean \pm SEM of 4 preparations for each group.

Effect of Ketanserin on 5-HT Induced Contractile Responses in Ureter from Young Pigs

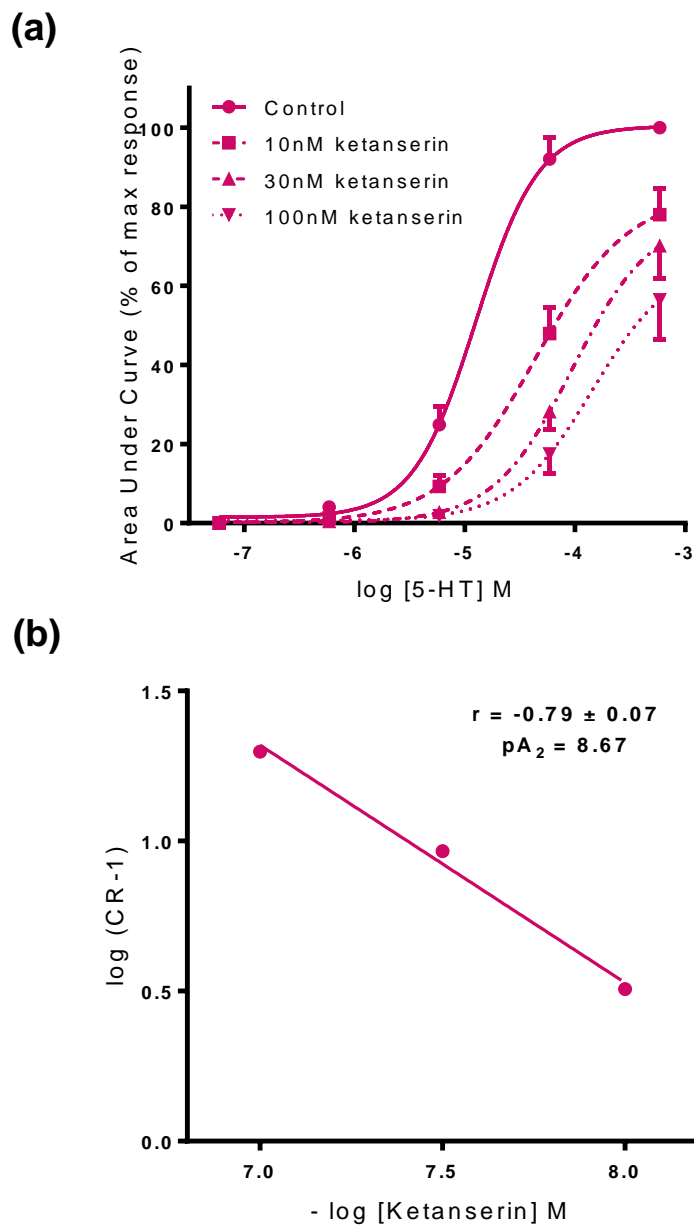


Figure 3.5 Concentration-response curves for 5-HT in the presence and absence of ketanserin (10 – 100nM) in isolated distal ureter from young pigs (a), with corresponding Schild plots for ketanserin (b). Responses are expressed as % of maximal response for AUC. Results are presented as mean \pm SEM of 8 preparations for each group.

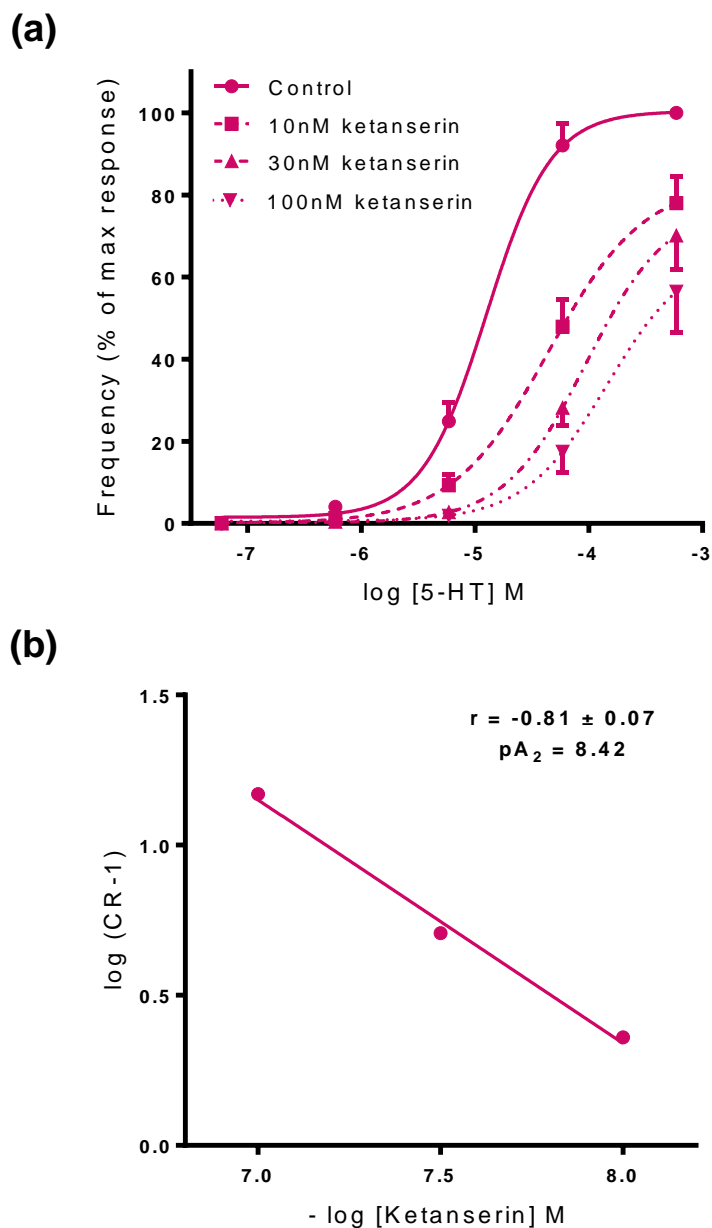
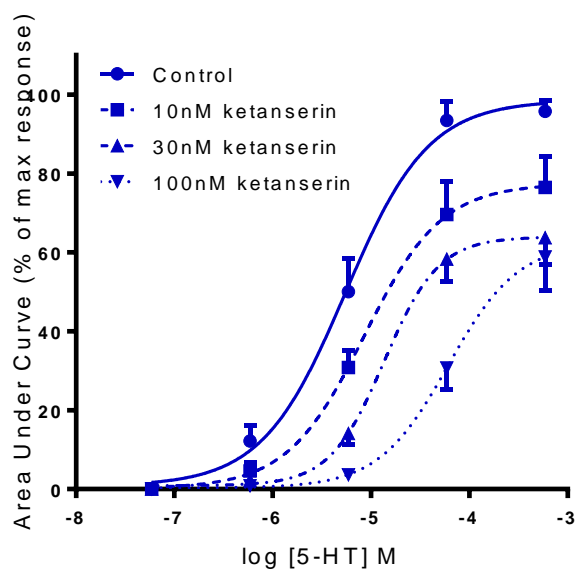


Figure 3.6 Concentration-response curves for **5-HT** in the presence and absence of **ketanserin (10 – 100nM)** in isolated distal ureter from young pigs (a), with corresponding Schild plots for ketanserin (b). Responses are expressed as % of maximal **frequency**. Results are presented as mean \pm SEM of 8 preparations for each group.

Effect of Ketanserin on 5-HT-induced Contractile Responses in Ureter from Old Pigs

(a)



(b)

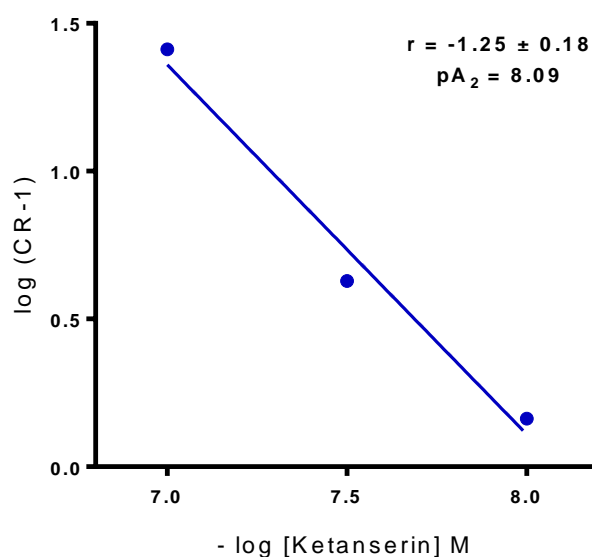
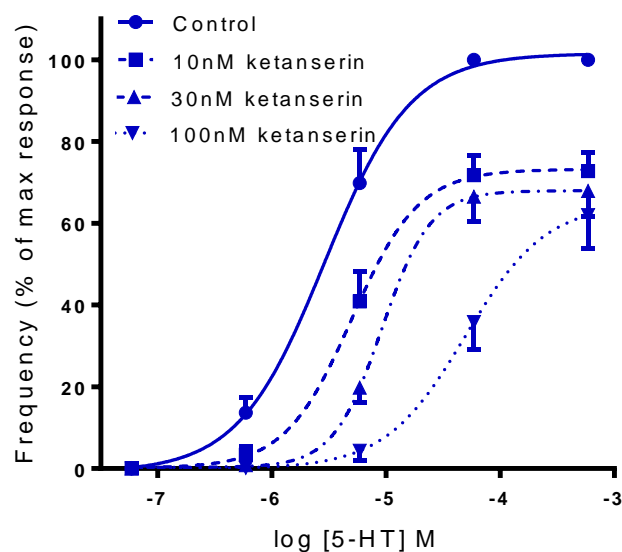


Figure 3.7 Concentration-response curves for 5-HT in the presence and absence of ketanserin (10 – 100nM) in isolated distal ureter from old pigs (a), with corresponding Schild plots for ketanserin (b). Responses are expressed as % of maximal response for AUC. Results are presented as mean \pm SEM of 8 preparations for each group.

(a)



(b)

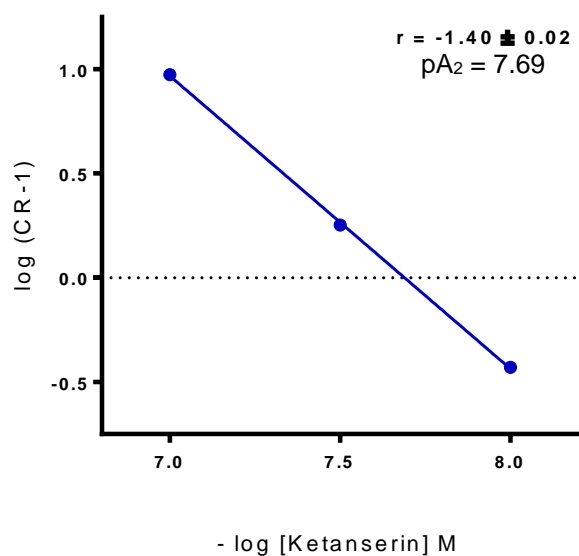


Figure 3.8 Concentration-response curves for **5-HT** in the presence and absence of **ketanserin (10 – 100nM)** in isolated distal ureter from old pigs (a), with corresponding Schild plots for ketanserin (b). Responses are expressed as % of maximal response for **frequency**. Results are presented as mean \pm SEM of 8 preparations for each group.

Effect of Ketanserin on α -Methyl-5-HT-induced Contractile Responses in Ureter from Young Pigs

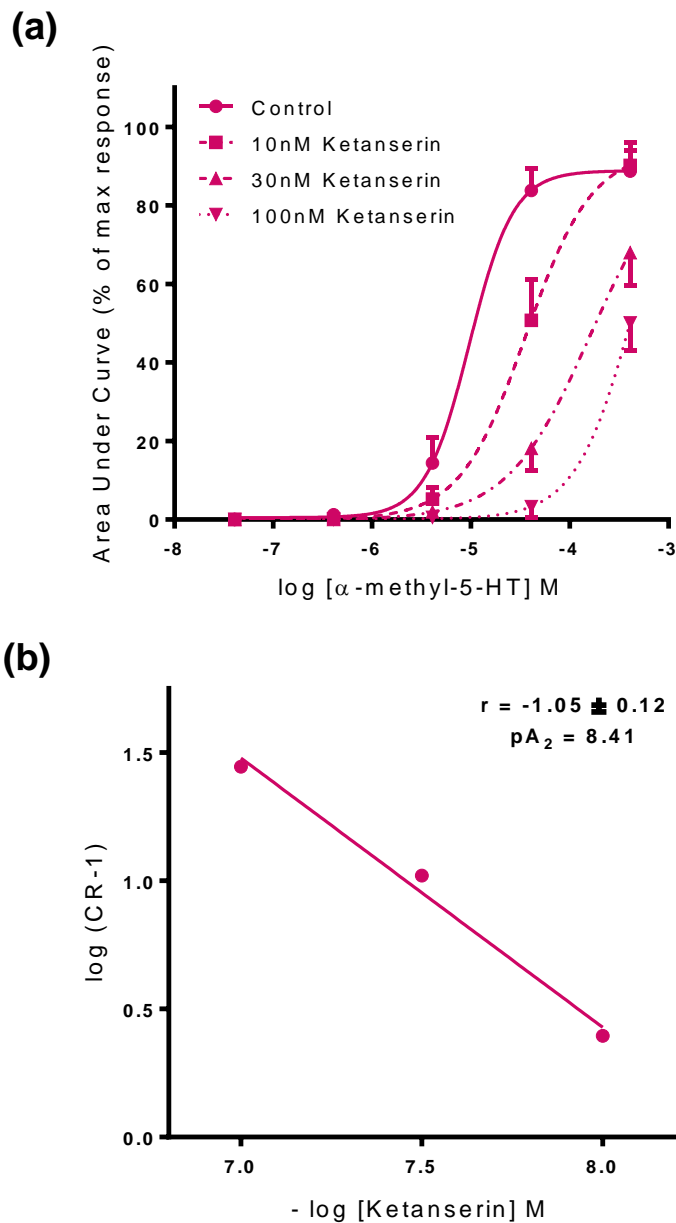


Figure 3.9 Concentration-response curves for α -methyl-5-HT in the presence and absence of ketanserin (10 – 100nM) in isolated distal ureter from young pigs (a), with corresponding Schild plots for ketanserin (b). Responses are expressed as % of maximal response for AUC. Results are presented as mean \pm SEM of 8 preparations for each group.

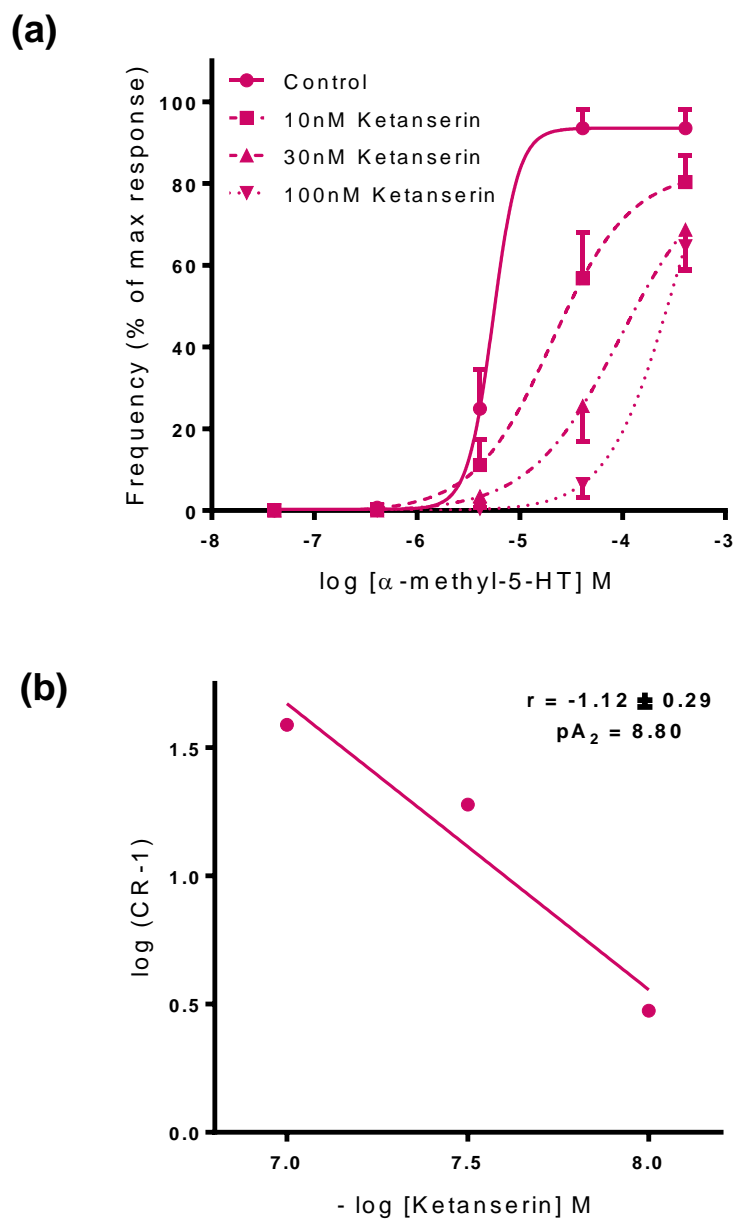


Figure 3.10 Concentration-response curves for α -methyl-5-HT in the presence and absence of ketanserin (10 – 100nM) in isolated distal ureter from young pigs (a), with corresponding Schild plots for ketanserin (b). Responses are expressed as % of maximal response for frequency. Results are presented as mean \pm SEM of 8 preparations for each group.

Effect of Ketanserin on α -Methyl-5-HT-induced Contractile Responses in Ureter from Old Pigs

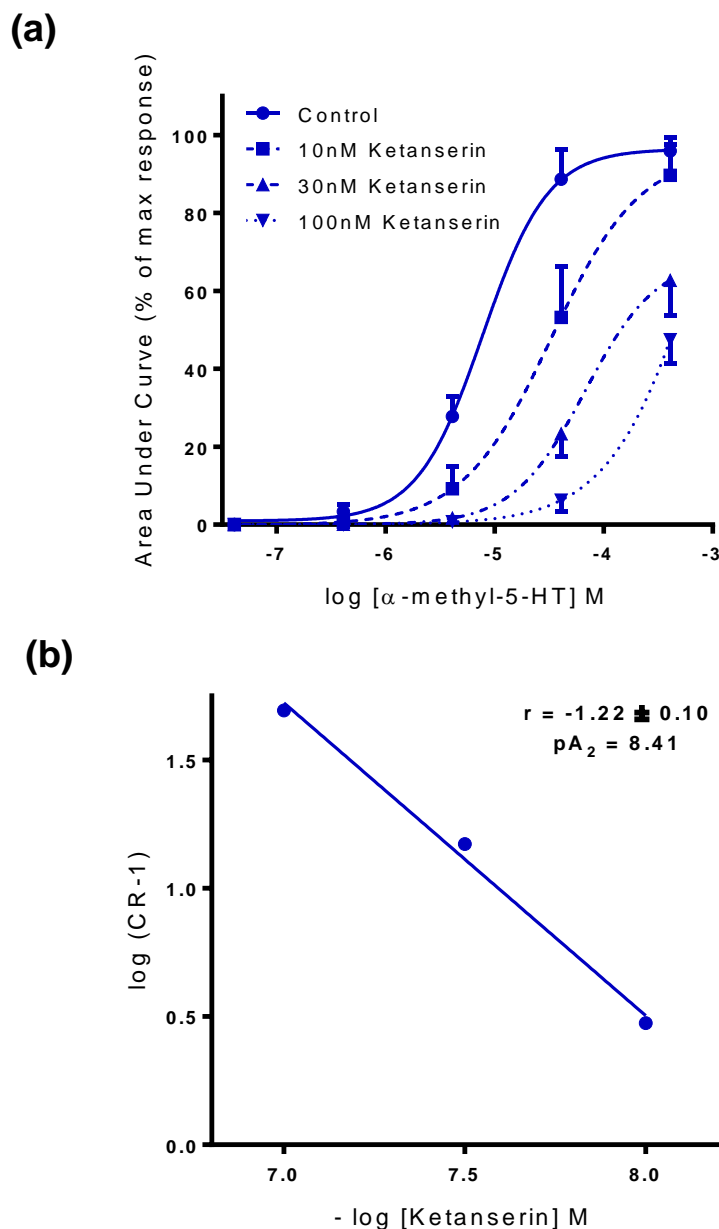


Figure 3.11 Concentration-response curves for α -methyl-5-HT in the presence and absence of ketanserin (10 – 100nM) in isolated distal ureter from old pigs (a), with corresponding Schild plots for ketanserin (b). Responses are expressed as % of maximal response for AUC. Results are presented as mean \pm SEM of 8 preparations for each group.

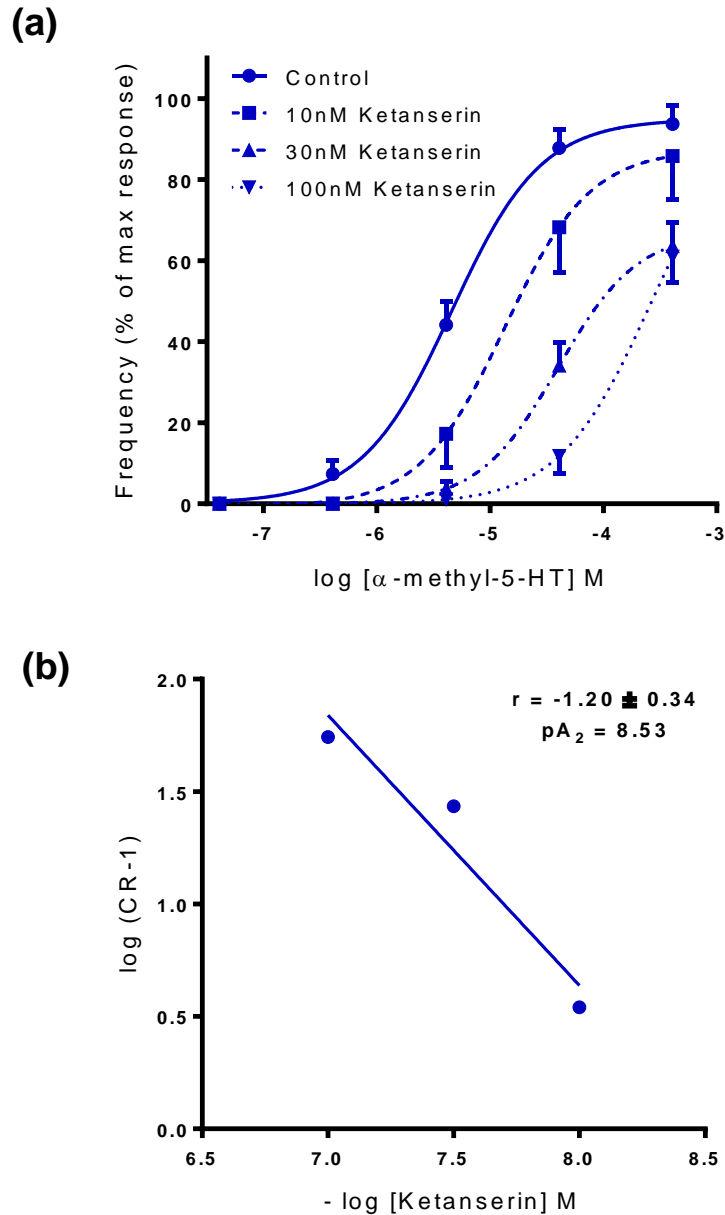


Figure 3.12 Concentration-response curves for α -methyl-5-HT in the presence and absence of ketanserin (10 – 100nM) in isolated distal ureter from old pigs (a), with corresponding Schild plots for ketanserin (b). Responses are expressed as % of maximal response for frequency. Results are presented as mean \pm SEM of 8 preparations for each group.

Ketanserin (10 – 100nM) was also effective in producing a rightward shift of concentration-response curves to α -methyl-5-HT in tissues from both age groups (Figures 3.9a, 3.10a, 3.11a and 3.12a). A comparison of concentration-response curve slopes also found no significant difference, supporting a competitive antagonism. Slopes of Schild plots were not significantly different from unity, also, supporting competitive antagonist mechanism at a single receptor subtype. pA₂ estimates are shown in Table 3.2 below.

Table 3.2 Affinity estimates for ketanserin with 5-HT and α -methyl-5-HT (*p<0.05 vs 5-HT-induced frequency of responses in young pig)

Agonist	Animal age	Response	pA ₂ estimate
5-HT	Young	AUC	8.67
		Frequency	8.42
	Old	AUC	8.09
		Frequency	7.69*
α -methyl-5-HT	Young	AUC	8.41
		Frequency	8.80
	Old	AUC	8.41
		Frequency	8.53

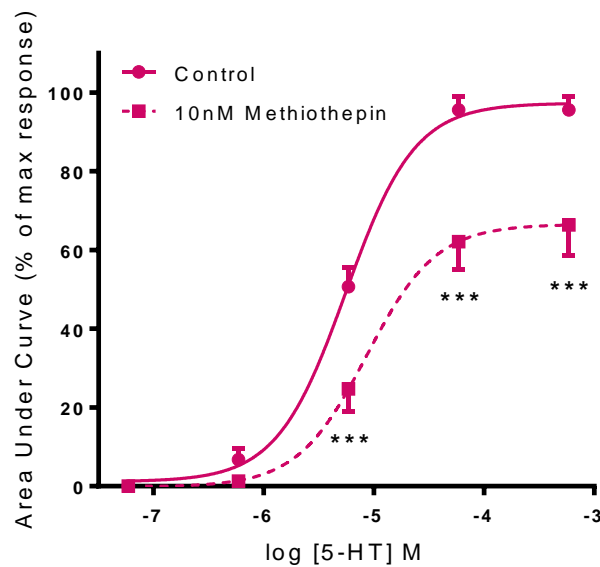
Effects of 5-HT Receptor Antagonists (Methiothepin, RS-10221, GF109203X, GR-113808, SB699551, and SB269970) on 5-HT-induced Contractile Responses

The 5-HT receptor antagonists RS-102221 (30nM, 5-HT_{2C} selective), ondansetron (30nM, 5-HT₃ selective), SB 699551 (10nM, 5-HT₅ selective), SB 399885 (100nM, 5-HT₆ selective) and SB 269970 (10nM, 5-HT₇ selective) did not affect maximal contractile responses to 5-HT (Figures 3.15 - 3.18 and 3.21 - 3.26) and pEC₅₀ for 5-HT were similar to control values (Table 3.3), in isolated tissues from both older and younger animals.

The non-selective 5-HT receptor antagonist methiothepin (10nM) significantly reduced maximal AUC responses to 5-HT in ureters from younger animals (Figure 3.13a, paired student's *t*-test, *p*<0.005) but not in older animals (Figure 3.14a, *p*>0.05). This effect was not observed for 5-HT responses expressed as frequency of phasic contractions (Figure 3.13b). Additionally, methiothepin also significantly shifted curves to the right and depressed pEC₅₀ values for 5-HT-induced contractile responses expressed as AUC in tissues from the younger animals but not in tissues from older animals (Table 3.3).

Comparatively, GR-113808, a selective 5-HT₄ antagonist (100nM) reduced maximal responses to 5-HT when expressed as AUC in tissues from older animals (Figure 3.20a, paired student's *t*-test, *p*<0.05) but not in younger animals (Figure 3.19a). Maximal responses to 5-HT expressed as frequency of phasic contractions were unaltered by GR-113808 (Figures 3.19b and 3.20b). pEC₅₀ values were also unaffected by 100nM GR-113808 (Table 3.3).

(a)



(b)

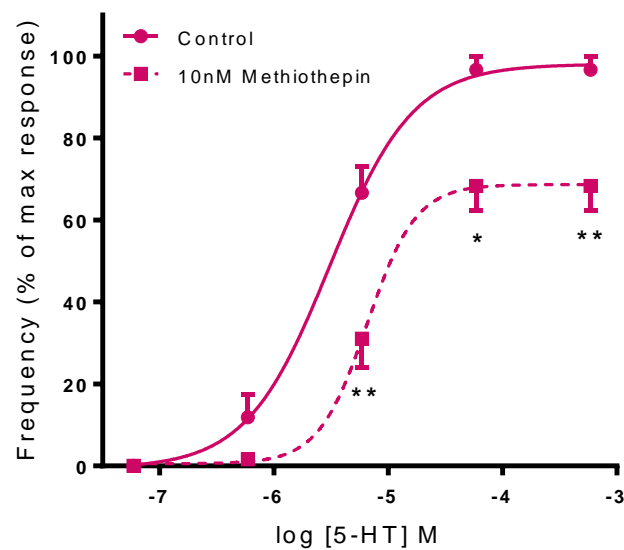
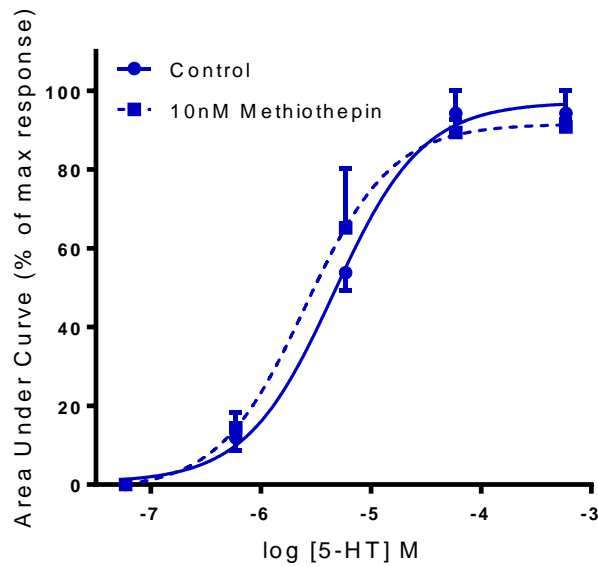


Figure 3.13 Concentration-response curves for 5-HT in the presence and absence of 5-HT antagonist **methiothepin (10nM)** in isolated distal ureter from young pigs. Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are presented as mean \pm SEM of 8 preparations for each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control).

(a)



(b)

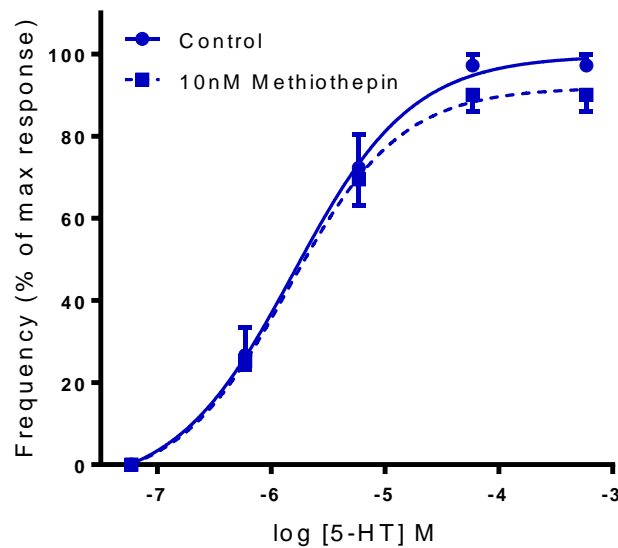


Figure 3.14 Concentration-response curves for 5-HT in the presence and absence of 5-HT antagonist **methiothepin (10nM)** in isolated distal ureter from old pigs. Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are presented as mean \pm SEM of 8 preparations for each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control).

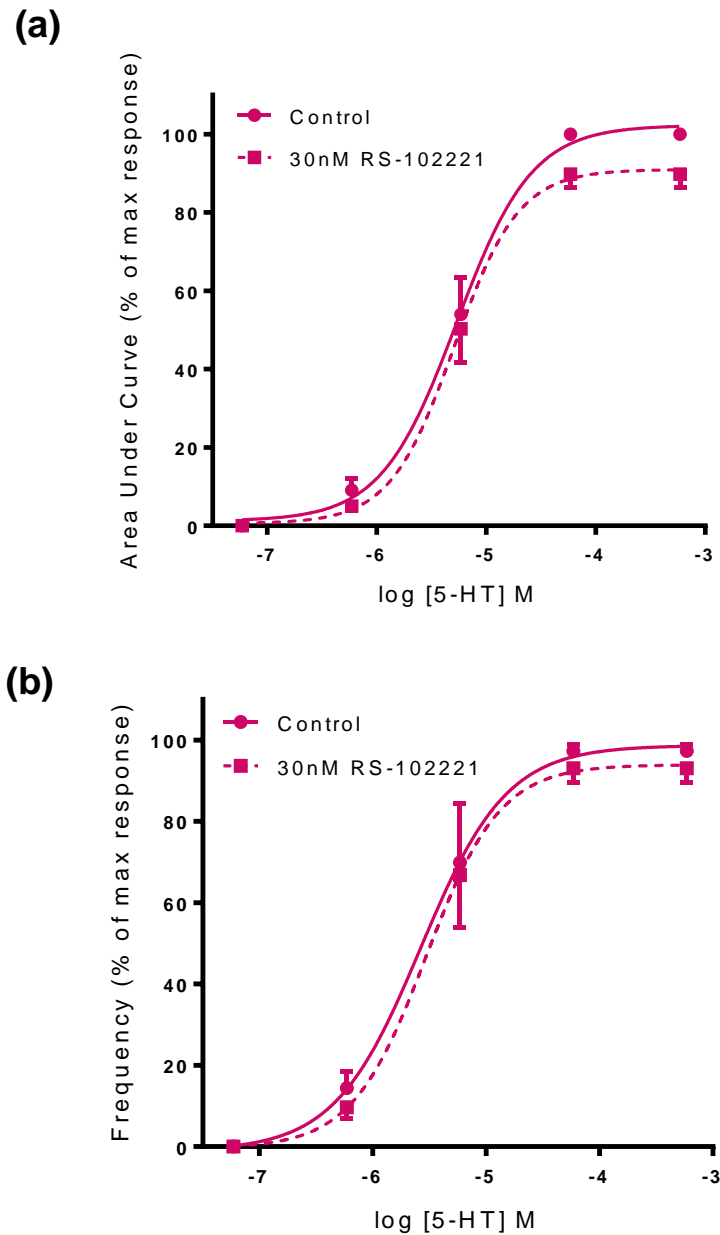


Figure 3.15 Concentration-response curves for 5-HT in the presence and absence of 5-HT_{2c} antagonist RS-102221 (30nM) in isolated distal ureter from young pigs. Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are presented as mean \pm SEM of 8 preparations for each group (* p <0.05, ** p <0.01, *** p <0.001 vs control).

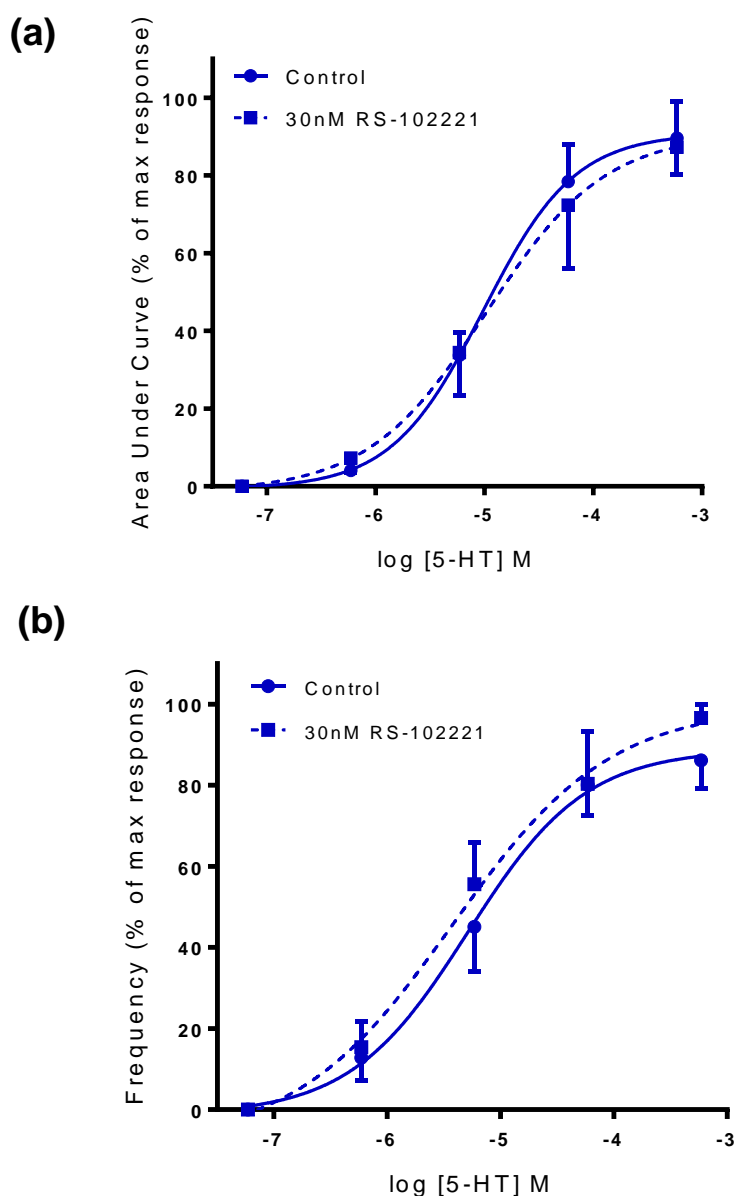
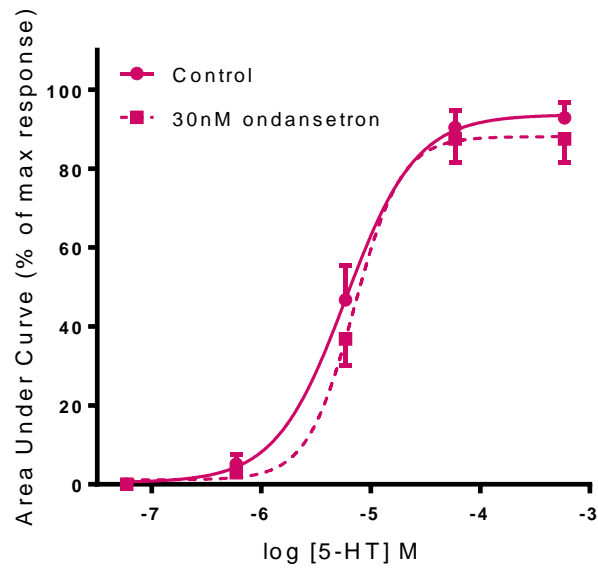


Figure 3.16 Concentration-response curves for 5-HT in the presence and absence of **5-HT_{2c} antagonist RS-102221 (30nM)** in isolated distal ureter from old pigs. Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are presented as mean \pm SEM of 8 preparations for each group (* p <0.05, ** p <0.01, *** p <0.001 vs control).

(a)



(b)

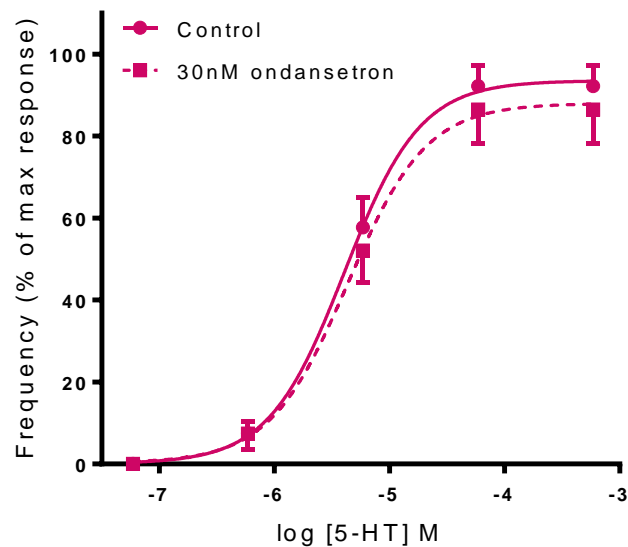
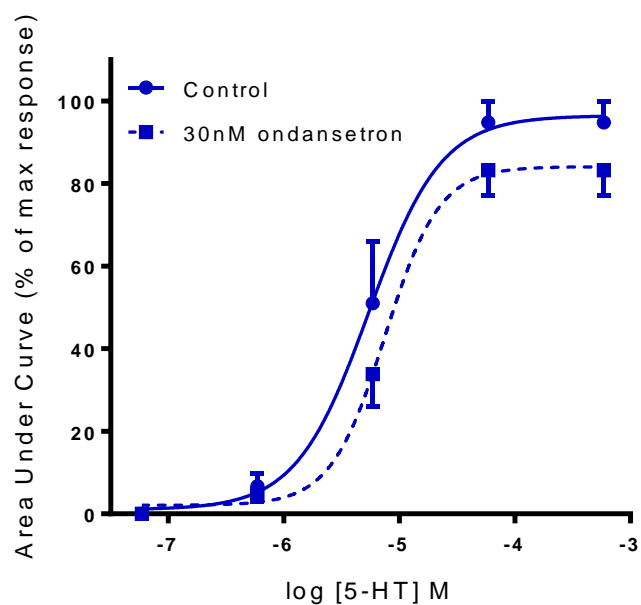


Figure 3.17 Concentration-response curves for 5-HT in the presence and absence of **5-HT₃ antagonist ondansetron (30nM)** in isolated distal ureter from young pigs. Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are presented as mean \pm SEM of 8 preparations for each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control).

(a)



(b)

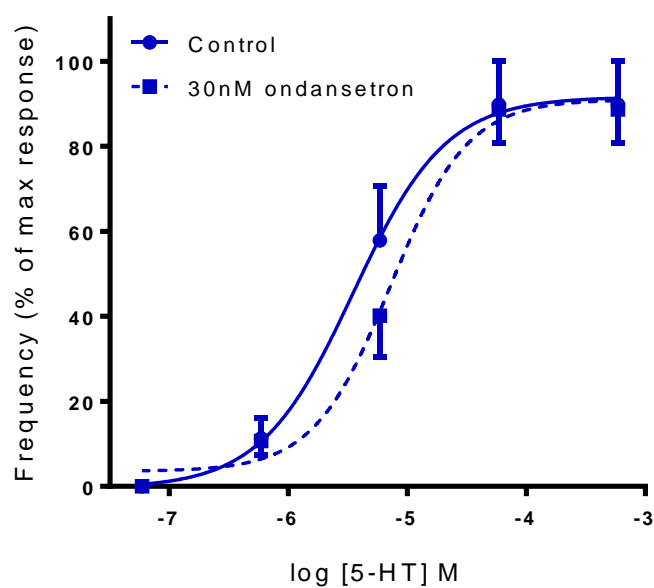
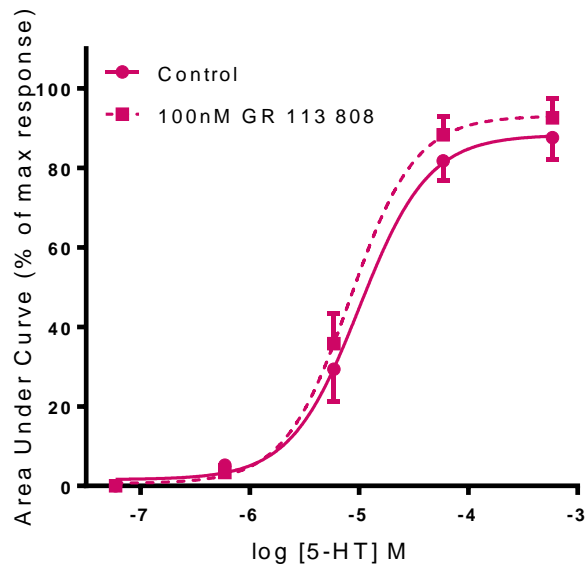


Figure 3.18 Concentration-response curves for 5-HT in the presence and absence of **5-HT₃ antagonist ondansetron (30nM)** in isolated distal ureter from old pigs. Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are presented as mean \pm SEM of 8 preparations for each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control).

(a)



(b)

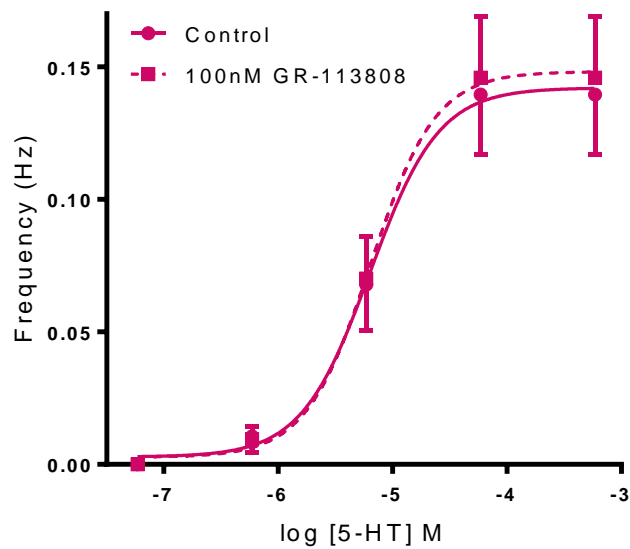


Figure 3.19 Concentration-response curves for 5-HT in the presence and absence of **5-HT₄ antagonist GR-113808 (100nM)** in isolated distal ureter from young pigs. Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are presented as mean \pm SEM of 8 preparations for each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control).

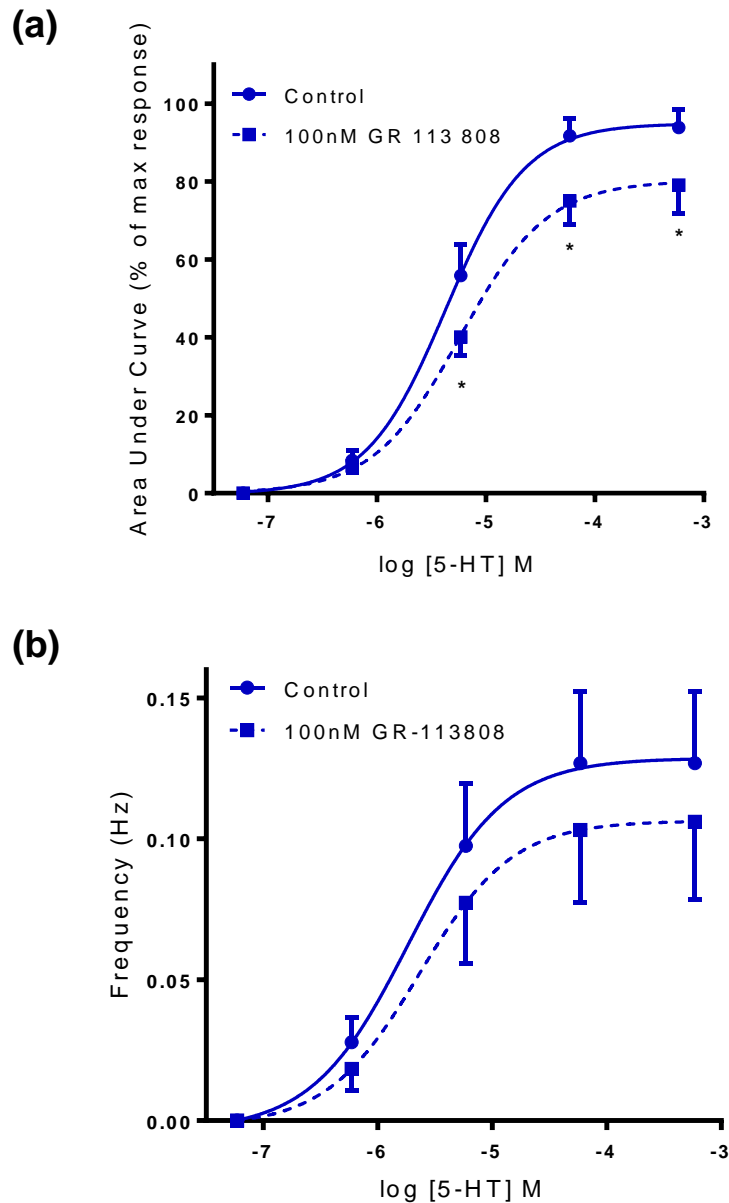
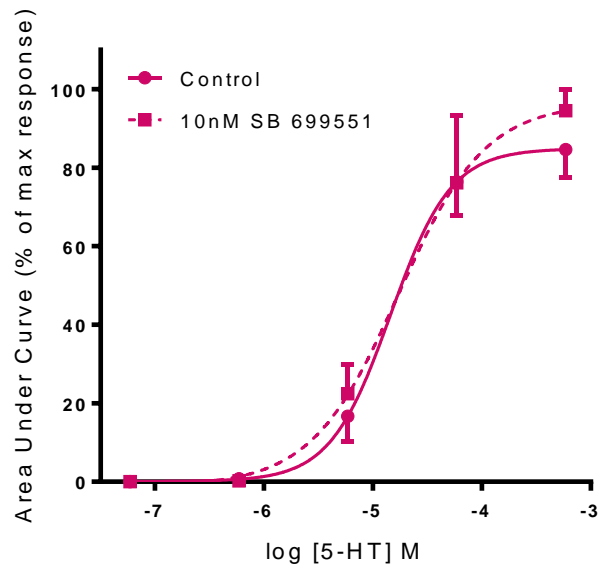


Figure 3.20 Concentration-response curves for 5-HT in the presence and absence of 5-HT₄ antagonist GR-113808 (100nM) in isolated distal ureter from old pigs. Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are presented as mean \pm SEM of 8 preparations for each group (* p <0.05, ** p <0.01, *** p <0.001 vs control).

(a)



(b)

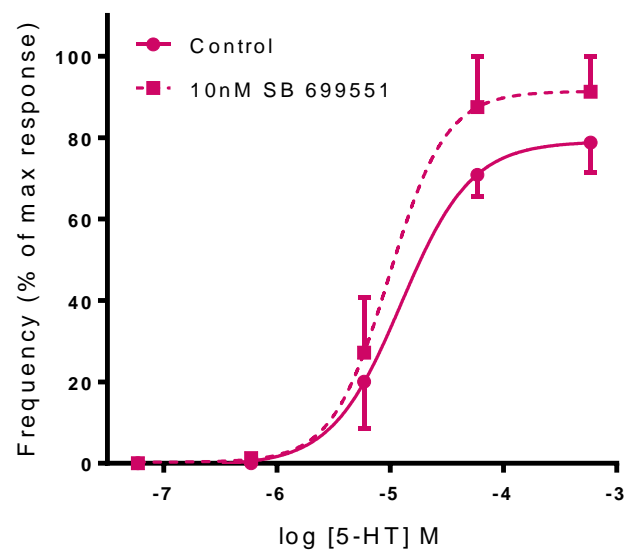


Figure 3.21 Concentration-response curves for 5-HT in the presence and absence of **5-HT₅ antagonist SB699551 (10nM)** in isolated distal ureter from young pigs. Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are presented as mean \pm SEM of 8 preparations for each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control).

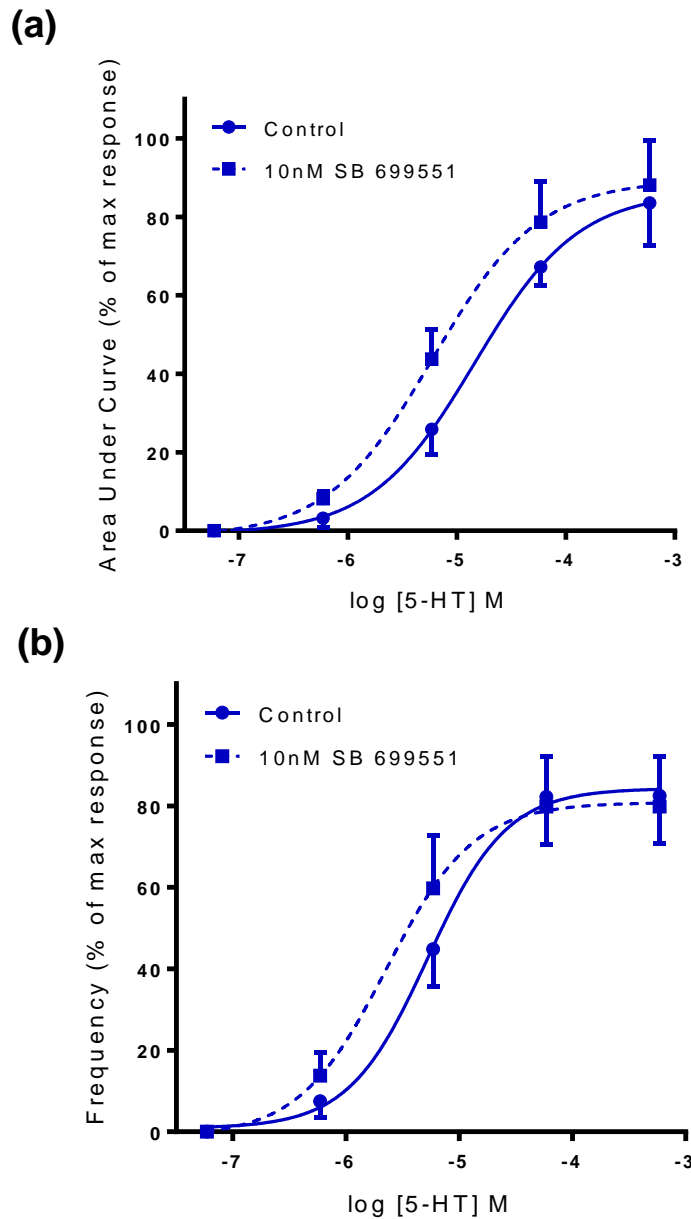


Figure 3.22 Concentration-response curves for 5-HT in the presence and absence of **5-HT₅ antagonist SB699551 (10nM)** in isolated distal ureter from old pigs. Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are presented as mean \pm SEM of 8 preparations for each group (* p <0.05, ** p <0.01, *** p <0.001 vs control).

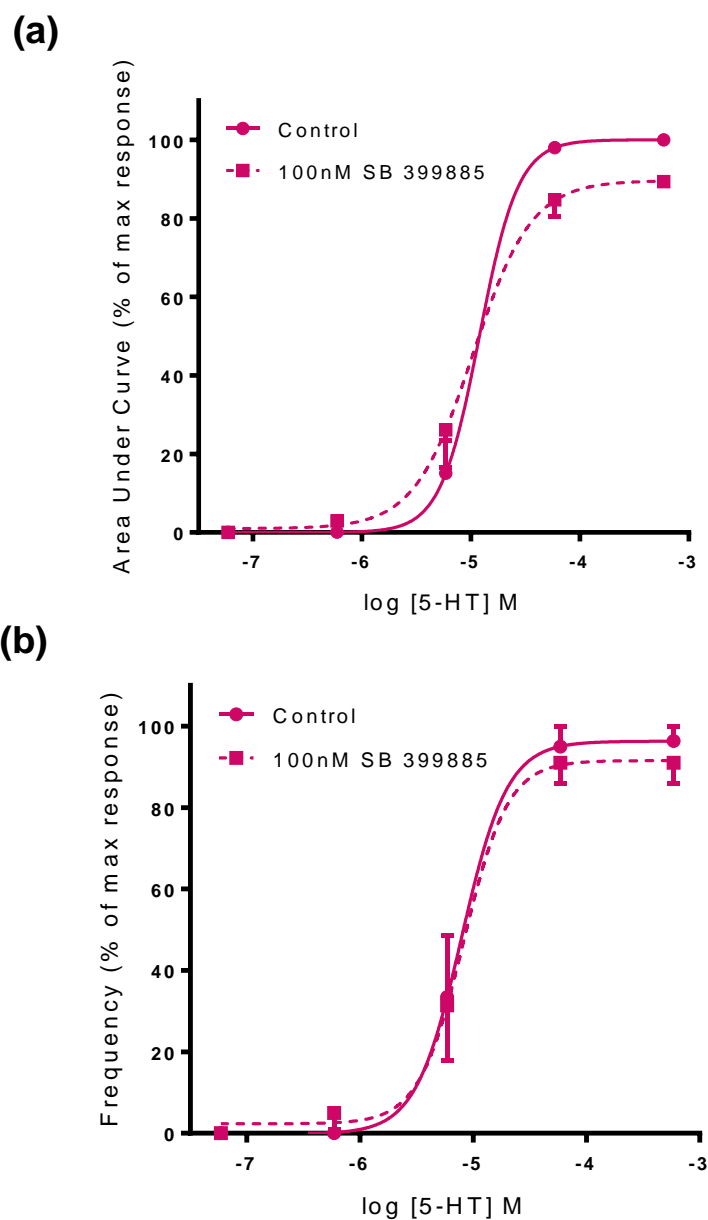


Figure 3.23 Concentration-response curves for 5-HT in the presence and absence of **5-HT₆ antagonist SB399885 (100nM)** in isolated distal ureter from young pigs. Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are presented as mean \pm SEM of 8 preparations for each group (* p <0.05, ** p <0.01, *** p <0.001 vs control).

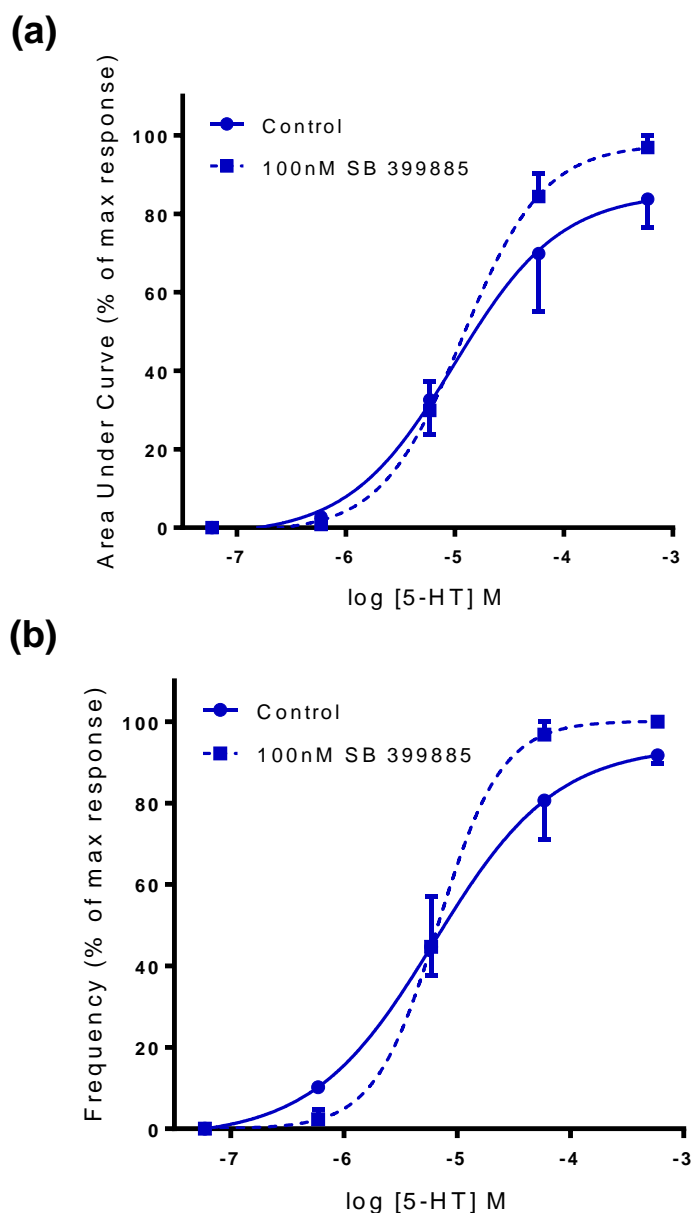
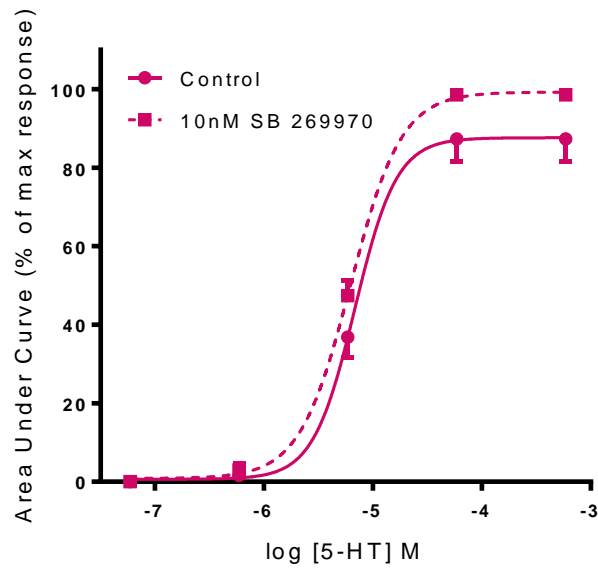


Figure 3.24 Concentration-response curves for 5-HT in the presence and absence of **5-HT₆ antagonist SB399885 (100nM)** in isolated distal ureter from old pigs. Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are presented as mean \pm SEM of 8 preparations for each group (* p <0.05, ** p <0.01, *** p <0.001 vs control).

(a)



(b)

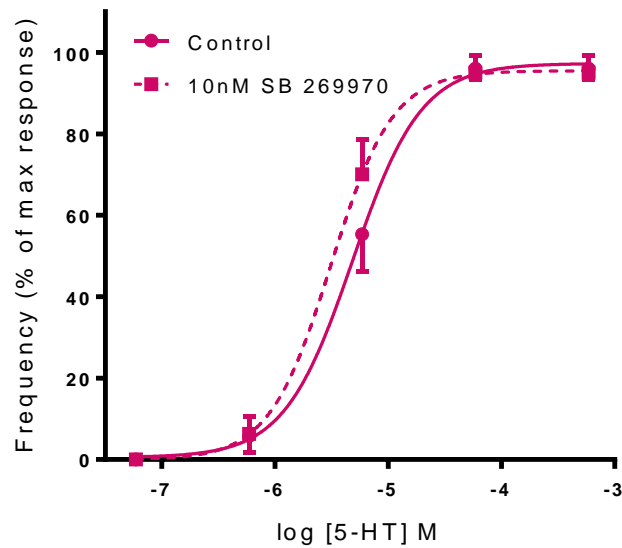


Figure 3.25 Concentration-response curves for 5-HT in the presence and absence of 5-HT₇ antagonist SB269970 (10nM) in isolated distal ureter from young pigs. Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are presented as mean \pm SEM of 8 preparations for each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control).

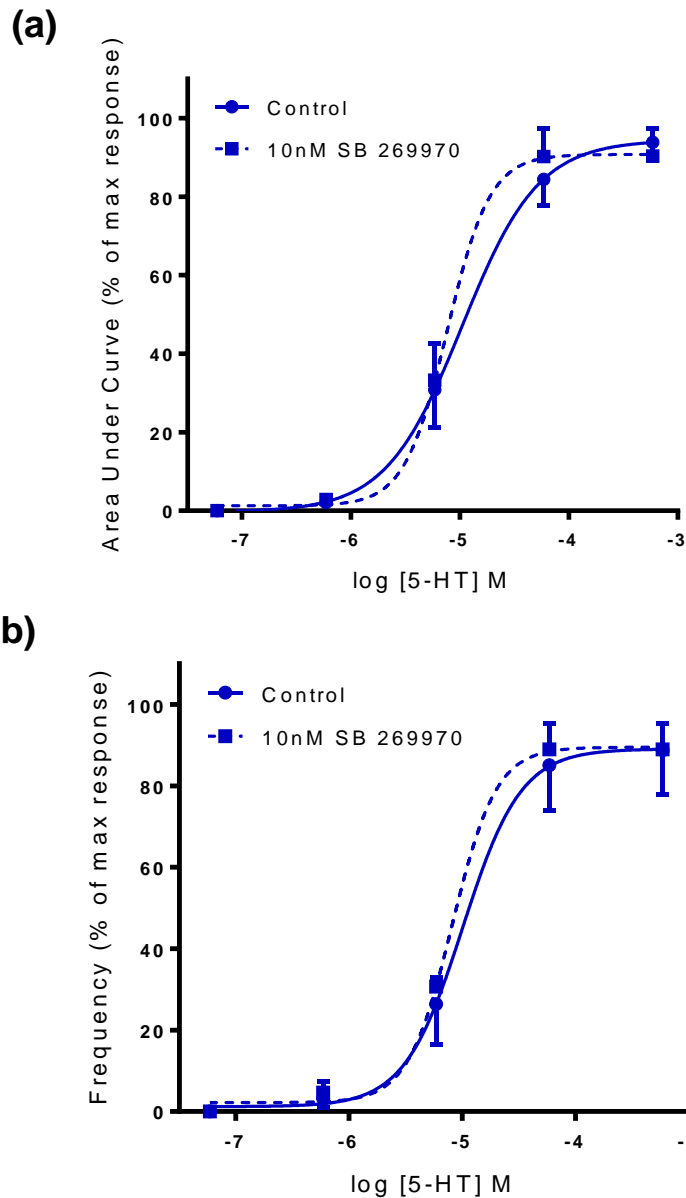


Figure 3.26 Concentration-response curves for 5-HT in the presence and absence of 5-HT₇ antagonist SB269970 (10nM) in isolated distal ureter from old pigs. Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are presented as mean \pm SEM of 8 preparations for each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control).

Table 3.3 (1) pEC_{50} values for 5-HT in the presence and absence of antagonists (continued on following page).

Antagonist	Group	Response	pEC_{50}	
			Absent	Present
10nM methiothepin (non-selective)	Young	AUC	5.26 ± 0.05	$4.72 \pm 0.05^*$
		Frequency	5.51 ± 0.09	5.19 ± 0.10
	Old	AUC	5.34 ± 0.06	5.60 ± 0.24
		Frequency	5.80 ± 0.15	5.83 ± 0.12
30nM RS-10221 (5-HT _{2c})	Young	AUC	5.27 ± 0.08	5.29 ± 0.07
		Frequency	5.58 ± 0.16	5.53 ± 0.14
	Old	AUC	5.01 ± 0.17	4.98 ± 0.31
		Frequency	5.29 ± 0.22	5.42 ± 0.13
30nM ondansetron (5-HT ₃)	Young	AUC	5.22 ± 0.08	5.15 ± 0.12
		Frequency	5.39 ± 0.10	5.36 ± 0.14
	Old	AUC	5.27 ± 0.12	5.12 ± 0.12
		Frequency	5.45 ± 0.21	5.14 ± 0.13
100nM GR-113808 (5-HT ₄)	Young	AUC	5.00 ± 0.12	5.08 ± 0.09
		Frequency	5.19 ± 0.17	5.19 ± 0.16
	Old	AUC	5.37 ± 0.10	5.23 ± 0.12
		Frequency	5.73 ± 0.36	5.63 ± 0.44
10nM SB 699551 (5-HT ₅)	Young	AUC	4.84 ± 0.15	4.77 ± 0.21
		Frequency	4.90 ± 0.18	5.01 ± 0.22
	Old	AUC	4.84 ± 0.19	5.22 ± 0.21
		Frequency	5.28 ± 0.18	5.65 ± 0.28

Table 3.3 (2) pEC_{50} values of 5-HT in the presence and absence of antagonists (continued from previous page).

Antagonist	Group	Response	pEC_{50}	
			Absent	Present
100nM SB 399855 (5-HT ₆)	Young	AUC	4.92 ± 0.15	4.98 ± 0.12
		Frequency	5.10 ± 0.22	5.09 ± 0.26
	Old	AUC	5.01 ± 0.25	4.93 ± 0.10
		Frequency	5.20 ± 0.16	5.17 ± 0.08
10nM SB 269970 (5-HT ₇)	Young	AUC	5.11 ± 0.11	5.21 ± 0.05
		Frequency	5.31 ± 0.08	5.51 ± 0.12
	Old	AUC	4.98 ± 0.12	5.12 ± 0.20
		Frequency	5.00 ± 0.21	5.09 ± 0.17

3.4 Discussion

Previously, it has been demonstrated in the majority of studies, that 5-HT stimulates contraction of smooth muscle in isolated ureteral strips in various species (Gidener et al., 1995, Gidener et al., 1999, Hernandez et al., 2003). In the current study, similarly, it was observed that 5-HT was able to concentration-dependently increase contractile activity when expressed as AUC or frequency in the distal ureter from older and younger pigs. This is in agreement with most other findings in tissues from pig and human (Gidener et al., 1995, Gidener et al., 1999, Hernandez et al., 2003) but not with a study performed in isolated rabbit ureter, where 5-HT did not induce any contractile response (Yalcin et al., 2013). 5-HT was able to concentration-dependently increase contractile responses in the porcine isolated distal ureter, which supports a study in the porcine intravesical ureter, where similar results were observed (Hernandez et al., 2003). However, in that study, 5-HT preferentially increased contractile tone. While phasic contractions developed in the presence of 5-HT, the frequency of these phasic contractions was not related by 5-HT, unlike in the present study (Hernandez et al., 2003). In addition to this, these authors found that phasic contractions were abolished during 5-HT-evoked increases in ureteral tone (Hernandez et al., 2003). Our data did not indicate any significant increase in baseline tone upon addition of either 5-HT or α -methyl-5-HT, at any concentration. This observed difference could be explained on the basis of experimental protocol, including tissue strips suspension methods, which was not specified in their report. Also, a difference in the ureteral region used may explain the difference in finding. The intravesical ureter region that was utilised in their experiment was proposed to be the ureteral component which acts as the sphincter to control movement of urine into the bladder (Blok et al., 1985, Hernandez et al., 1999, Prieto et al., 1997). The ureteral region utilised in the present study was the distal ureter which is located proximal to the intravesical

ureter. Thus, it is suggested that 5-HT may play different, region specific roles – more related to the closure of the ureteral tube at the intravesical ureter to prevent vesicoureteral reflux, whereas at the distal region 5-HT receptor stimulation plays a significant role in development of phasic contractions for ureteral peristalsis.

In the present study, isolated tissues showed similar responsiveness to both 5-HT and α -methyl-5-HT (a 5-HT₂ selective agonist), and also in tissues from both age groups. This supports other reports where α -methyl-5-HT was also shown to be potent in stimulating contractile responses in pig intravesical ureter, although no comparisons to 5-HT-mediated contractile responses were performed in those studies (Hernandez et al., 2003, Humphrey, 1984).

Ketanserin, the selective 5-HT_{2A} antagonist, was capable of shifting concentration-response curves at 10 – 100nM, suggesting the role for 5-HT_{2A} in mediation of 5-HT-induced contractions. The slopes of Schild plots for ketanserin with both 5-HT and α -methyl-5-HT were not significantly different from unity in tissues from the younger animals, suggesting that 5-HT_{2A} is the sole receptor subtype responsible for these 5-HT-induced contractions. Additionally, in tissues from the younger animals, affinity estimates from antagonism of responses by ketanserin were comparable to values reported at the 5-HT_{2A} receptor in the literature (8.7) (Bonhaus et al., 1995). In tissues from young animals, there was a slight rightward shift of the 5-HT concentration-response curve in the presence of methiothepin (10nM), suggesting again, the involvement of the 5-HT_{2A} receptor subtype. While methiothepin is generally a non-selective 5-HT antagonist, its affinity is highest at the 5-HT_{2A} receptor subtype (Hoyer et al., 1994). Furthermore, the lack of effect on 5-HT concentration response curves by all other 5-HT receptor antagonists, also suggests that effects of 5-HT are mediated via activation of the 5-HT_{2A} receptor in isolated ureter from younger animals.

In 5-HT-induced frequency responses in tissues from the older animals, competitive antagonism by ketanserin was not observed. The Schild plot slope was significantly greater than unity indicating additional actions of ketanserin on 5-HT-stimulated frequency responses in ureters from the older animals. The affinity estimate of 7.69 obtained from the intercept of the Schild plot for frequency responses is most likely not reflective of the true affinity value for ketanserin. Additionally, the affinity estimate for AUC responses to 5-HT in tissues from the older animals was low (8.0) compared to affinity value for in the literature (Bonhaus et al., 1995) and the affinity estimate from younger animals. Additionally, the rightward shift of the 5-HT response curves in the tissues from the younger animals when incubated with methiothepin (10nM), was not observed in the older animals. This suggests the possibility of methiothepin having another unidentified action on the frequency of phasic contractions in the ureter of older animals in response to 5-HT, masking the effects of 5-HT_{2A} receptor subtype inhibition. Affinity estimates for ketanserin at 5-HT_{2A} receptors with α -methyl-5-HT stimulation in older animals were also comparable to values (8.7) found in the literature (Bonhaus et al., 1995), indicating that the additional effect of methiothepin is unlikely to be via 5-HT_{2B} or 5-HT_{2C} receptor subtypes. This is further supported by the lack of inhibition of 5-HT contractile response by the 5-HT_{2C} selective antagonist, RS-10221 (30nM). Although there was a decrease in maximal response in the presence of GR-113808, a selective 5-HT₄ receptor antagonist, it would be imprecise to deduce that this is the additional functional receptor subtype in the older animals, as there was no significant change in pEC₅₀ values in the presence of this antagonist. Therefore, we suggest that in the younger animals, the 5-HT-induced contractile response is due to stimulation of the 5-HT_{2A} receptor subtype, whilst in the older animals, 5-HT_{2A} receptor is responsible for the contractile effect of 5-HT and the stimulation of 5-HT on another receptor could cause smooth muscle relaxation. This is also a possible explanation for our preliminary results,

where 5-HT contractile responses were depressed in isolated tissues from older animals (Chapter 2).

In the isolated human (Gidener et al., 1999) and pig ureter (Hernandez et al., 2003), it has been shown that a slight contractile response develops in response to 5-CT, a selective 5-HT_{1A} receptor subtype agonist. We did not perform any selective antagonist experiments to investigate 5-HT₁ receptors due to the lack of availability of selective 5-HT₁ antagonists. In the latter study, there was a lack of effect on the 5-CT-induced responses by selective 5-HT_{1A/1B} antagonist, indicating that these receptors are not involved in the 5-CT-induced tone and the response maybe could be due to activation of a different mechanism (Hernandez et al., 2003).

Despite functional studies showing the significant role of 5-HT in ureteral contractility, there is still no agreement as to whether 5-HT is released endogenously in the ureter. Identifying the source of 5-HT in the ureter is an essential task for further understanding of the physiological role of 5-HT-induced ureteral contraction. However, this is proving a difficult task, as to our knowledge, the existence of 5-HT-containing or 5-HT-producing neurons has not yet been reported. It is presumed that platelets and mast cells might be the source of 5-HT in the ureter, as 5-HT-containing enterochromaffin cells that are found in the gut have not been identified in the ureter (Theoharides et al., 1982, Nocito et al., 2007, Ripoche, 2011).

It was previously demonstrated in the rabbit ureter that 5-HT-stimulated contractile responses are enhanced in the presence of ureteral obstruction (Yalcin et al., 2013). In the situation where intra-ureteral pressure may increase due to the presence of calculi, it is possible that capillaries could be damaged, leading to leakage of blood. Besides this, increased vascular permeability secondary to inflammation caused by ischemia could also occur, leading to blood leakage. In either case, 5-HT present in platelets and free 5-HT in plasma could permeate ureteral tissue. Mast cells,

playing a major role in the inflammatory process have been observed in all layers of the ureteral wall in the porcine ureter under normal conditions (Jerde et al., 2000, Vodenicharov et al., 2005). It is suggested that mast cells present in the ureter are most likely involved in maintaining local homeostasis and also play an important role in regulation of ureter motility through the release of transmitters including histamine and 5-HT in inflammatory circumstances. While our preliminary data (Chapter 2) demonstrated the lack of response of isolated ureteral strips to increasing concentrations of histamine, it is possible that this mast cell regulatory mechanism occurs via 5-HT. Additionally, there is also the possibility of an up-regulation of 5-HT_{2A} receptors in the ureter in obstructed states (Yalcin et al., 2013), although this requires study of receptor expression for confirmation.

3.5 Conclusion

To our knowledge, this is the first report to pharmacologically characterize, with Schild plots, the 5-HT receptor subtype/s mediating ureteral contractile responses to 5-HT. We are also the first to determine the age-related changes in these contractions. Although further studies are required to confirm changes in receptor expression, our functional characterization suggests that in younger animals, the 5-HT_{2A} receptor subtype is solely responsible for 5-HT-stimulated contractile responses of the isolated distal ureter. In older animals, we suggest that the 5-HT_{2A} receptor also plays a mediatory role, but the role appears to be more complicated than in the younger animals. In the literature, the 5-HT receptor-mediated pathway seems to be upregulated following ureteral obstruction, and therefore, this system could prove to be an effective target for treatment of ureteral calculi (Yalcin et al., 2013). Additionally, urinary side effects are not common in the prescription of 5-HT antagonists. However, there are rare reports of urinary retention as a side effect of this class of drugs (Verhamme et al., 2008).

CHAPTER 4:
The role of Rho-kinase pathway in
ureteral responses

4.1 Introduction

Normal ureteral contractility relies on effective contraction and relaxation of the smooth muscle cells which constitute the bulk of the ureteral wall. Skeletal and cardiac muscle cells are regulated by calcium through the tropomyosin-troponin complex. However, the contraction and relaxation of smooth muscle cells of the urinary tract is mediated by the enzymes myosin light chain kinase (MLCK) and its counteracting enzyme, myosin light chain phosphatase (MLCP), involved in myosin light chain (MLC) phosphorylation and dephosphorylation respectively (Zhang and DiSanto, 2011). It is well established that the changes in intracellular calcium as a trigger for contraction plays a significant role in various smooth muscle-related diseases and disorders. Consequently, the ability to manipulate and identify other aspects of intracellular signalling regulation of myosin activity and degree of muscle tone is under investigation.

It has been known since 1946, that calcium is the intracellular trigger for smooth muscle contraction, and that this is activated by an elevation of intracellular cytosolic calcium, $[Ca^{2+}]_i$ (Heilbrunn and Wiercinski, 1947). However, more recent research has demonstrated clearly that smooth muscle contraction can occur even in the absence of changes in $[Ca^{2+}]_i$. The major mechanism through which this occurs is the inhibition of smooth muscle MLCP via a guanine nucleotide-binding protein (G-protein)-coupled mechanism involving the enzyme Rho-kinase (ROCK). This process currently is defined as the 'calcium sensitization' pathway (Zhang and DiSanto, 2011, de Godoy and Rattan, 2011). The vital hallmark of calcium sensitization is the ability of smooth muscle to sustain contractile responses in the presence of low $[Ca^{2+}]_i$ (Kitazawa and Somlyo, 1991), but it has been shown that this pathway is also able to induce contraction of smooth muscle without necessarily altering $[Ca^{2+}]_i$ (Litten et al., 1987, de Godoy and Rattan, 2011). Since then, research has highlighted the

relevance of calcium sensitization which depends on the type of stimulation experienced by the myocyte (e.g. stimulation via different receptors).

The major enzyme involved in the calcium sensitization pathway is the ROCK enzyme and there are presently two known isoforms of ROCK, ROCK-II (ROCK α) and ROCK-I (ROCK β). Currently, the complete functional differences between these isoforms have still not been elucidated (Zhang and DiSanto, 2011, de Godoy and Rattan, 2011). ROCK is a specific enzyme activated via G-protein coupled receptors by the monomeric GTP-binding protein RhoA (Christ and Andersson, 2007). The Rho-proteins belong to the Ras superfamily of GTPases and there are three isoforms: RhoA, RhoB and RhoC (Rossman et al., 2005). Although these three isoforms appear to share approximately 80% amino acid homology, their cellular functions seem to be distinct with the most studied being RhoA (Sah et al., 2000). It is the RhoA isoform that is involved in smooth muscle contraction, but it is important to note that it has additional functions including regulation of cellular processes involving stress fiber formation, cell proliferation, migration and apoptosis (Arner and Pfitzer, 1999, Sah et al., 2000).

The binding of RhoA to ROCK causes a conformational change in the enzyme and also its subsequent autophosphorylation, increasing ROCK activity (Amano et al., 2000, Chen et al., 2002). Smooth muscle contraction is achieved by phosphorylation of MLCP by ROCK. MLCP is comprised of three subunits MYPT1, PP1c, and a small subunit whose function is still unclear (Borman et al., 2002). MYPT1 has been shown to be ubiquitously expressed, with a significantly higher concentration in smooth muscle cells (Christ and Andersson, 2007). Inhibition of MLCP is achieved by the phosphorylation of its MYPT1 subunit, which is a crucial substrate for ROCK (Kimura et al., 1996, Schmidt et al., 1999). The phosphorylation of MYTP1 subsequently suppresses PP1c activity,

inhibiting MLCP action and hence, results in a maintenance of MLC phosphorylation (Somlyo and Somlyo, 2000, Feng et al., 1999). Besides that, ROCK has also been suggested to be able to further increase calcium sensitization by phosphorylation of a PKC-potentiated phosphatase inhibitor, CPI-17. CPI-17 is a downstream target of PKC, which subsequently plays a vital role in smooth muscle contraction (Li et al., 1998).

In light that ROCK is able to stimulate smooth muscle contraction, a number of ROCK inhibitors have been developed and used as an effective approach to manipulate calcium sensitivity in smooth muscles. With the utilization of high throughput screening structural data is under minute investigation to identify a set of potential ROCK inhibitors (Takami et al., 2004). The two most researched ROCK inhibitors are Y-27632 and fasudil (HA-1077). Initially, fasudil was characterised as an intracellular Ca^{2+} antagonist (Asano et al., 1989, Satoh et al., 2001) whereas Y-27632 was developed as a smooth muscle relaxant and it was only later shown to dampen ROCK activity (Ishizaki et al., 2000, Uehata et al., 1997). These ROCK inhibitors have been shown to be effective in relieving symptoms of several diseases including vasospastic angina (Shimokawa, 2002, Hirooka and Shimokawa, 2005), renal disease (Sharpe and Hendry, 2003, Jan Danser, 2004) and pulmonary hypertension (Nagaoka et al., 2005, Abe et al., 2004).

Within the lower urinary tract, there have been many studies to determine the critical role of ROCK in normal bladder function. Similar to the ureter, the normal function of the bladder is also dependent on coherent mediation of detrusor smooth muscle contraction and relaxation. While it was initially believed that M_3 receptor stimulation induced contraction purely via phosphoinositide hydrolysis (Andersson et al., 1991, Harriss et al., 1995), it was later suggested that ROCK activation was also involved (Jezior et al., 2001). In the rat detrusor, it was found that Y-27632 and

fasudil both inhibited contractions evoked by carbachol but without affecting KCl-induced contractions (Wibberley et al., 2003). In line with this, a study on the human detrusor confirmed that carbachol-induced contractions mediated by M_3 receptors are largely depend on Ca^{2+} entry through nifedipine-sensitive channels together with activation of the ROCK pathway (Schneider et al., 2004). Furthermore, inhibition of each of these pathways was equally effective in inhibiting carbachol-induced contractile responses (Kirschstein et al., 2014). Besides that, Y-27632 also demonstrated the ability to suppress spontaneous tone and responses to the P2X receptor agonist α,β -methylene-ATP of the rabbit urinary bladder (Wibberley et al., 2003).

In contrast to the research into the significance of ROCK activity in bladder smooth muscle contraction, only a limited number of studies have focused on its importance in the ureter. In the normal rat ureter, it was shown that Y-27632, fasudil and H-1152 (another ROCK inhibitor) were all able to significantly decrease phasic contractions and Ca^{2+} transients (Shabir et al., 2004). This indicates that ROCK inhibition could modulate phasic contractions in the absence of calcium changes, which suggests a role for ROCK in calcium sensitization in the ureteral smooth muscle. However, this was not evident in the guinea-pig ureter, indicating there are species differences (Shabir et al., 2004). Furthermore, rho-kinase inhibitors fasudil and Y-27632 were shown to inhibit spontaneous contractions in the isolated guinea-pig and sheep ureter but not in the rat ureter (Levent and Buyukafsar, 2004, Shabir et al., 2004). In addition to this, another study on the sheep ureter demonstrated the presence of both isoforms of ROCK, ROCK-I and ROCK-II, and its mediation of agonist- and electrical field stimulated(EFS)-induced contractions as well as spontaneous contractile activity (Levent and Buyukafsar, 2004). Immunohistochemistry and immunoblotting studies showed the presence of both ROCK isoforms in the human ureter, and functional analysis supports this, where

spontaneous and EFS-induced contractile responses were depressed by Y-27632 in a concentration-dependent manner (Hong et al., 2005).

It has been proposed that ROCK-mediated contractile mechanisms could be extensively altered in response to pathological insults. For instance, in bladders of rabbits with chemically induced diabetes, upregulation of ROCK and CPI-17 was evident, resulting in an increase in MLC phosphorylation in the detrusor muscle, which was depressed by Y-27632 (Chang et al., 2006). Furthermore, in the male rabbit and rat bladder, an elevation in expressions of ROCK-I and ROCK-II was observed several weeks post experimental bladder outlet obstruction (Lin et al., 2008, Takahashi et al., 2009). In both these studies, it was also demonstrated that there was an increase in the sustained component of the detrusor contraction, especially in agonist-induced contractions to phenylephrine and carbachol (Lin et al., 2008, Takahashi et al., 2009). Similar to bladder outlet obstruction, unilateral ureteral obstruction was also shown to enhance expressions of ROCK-I and ROCK-II in the rabbit ureter in comparison to expression in non-obstructed ureters (Turna et al., 2007). To further support this finding, functional analysis revealed that contractile responses induced by electrical field stimulation, KCl, phenylephrine and carbachol in ureteral strips from rabbits with ureteral obstruction were significantly enhanced in comparison to those from control rabbits (Turna et al., 2007). Furthermore, Y-27632 was able to significantly suppress these contractile responses in both unilaterally obstructed and control ureters from the rabbit and also reduced contractions in the obstructed ureter to control levels (Turna et al., 2007).

In addition to alterations seen with pathology, ageing has been shown to alter contractile response in the detrusor strips of the bladder. With regards to the ROCK pathway, it was found that in the adult guinea pig bladder, agonist-induced contractions of detrusor strips were sensitive to blockade with Y-27632, whilst this inhibitor had a negligible effect on

tissue strips from older animals, thus suggesting the possibility of alteration in ROCK expression and/or activity with age (Gomez-Pinilla et al., 2008). Furthermore, in the human bladder, there was a strong correlation between inhibition of carbachol-induced contractions by Y-27632 with age, suggesting that ROCK-mediated carbachol-induced contractions are strongly age-dependent and may play a major role in ageing patients (Kirschstein et al., 2014).

In the literature, there are no studies reporting on the effect of age in the ROCK pathway in the ureter. It is well established that the α_1 -adrenergic receptor plays a major role in mediation of smooth muscle contraction in the ureter and as a G-protein coupled receptor, we hypothesised that the ROCK pathway may play some part in the regulation of ureteral smooth muscle contraction mediated by stimulation of this receptor. In addition to this, in previous chapters (Chapters 2 and 3), we have also discovered that 5-HT is an effective agonist to stimulate smooth muscle contraction in the ureter and, verified that 5-HT_{2A} is the receptor subtype modulating this contractile response in both old and young pigs. The receptor subtype, 5-HT_{2A} is also a G-protein coupled receptor and therefore, ROCK may also play a role in 5-HT-induced smooth muscle contractile response. The aim of this study was, therefore, to investigate the effect of the ROCK inhibitors, Y-27632 and fasudil, on phenylephrine- and 5-HT-stimulated contractile responses of isolated distal ureter from young and old animals, to determine if there are any changes in the ROCK pathways related to ageing. In addition to this, we also studied the levels of ROCK activity in the presence and absence of aforementioned agonists.

4.2 Methods and Materials

Tissue preparation and *in vitro* functional studies were performed as described in Section 2.1. For the first part of the experiment, isolated ureteral tissue strips were paired where one strip acted as the control while the other was incubated for 30 minutes with one of the following concentrations of ROCK inhibitors: 10 μ M Y-27632, 10 μ M fasudil, 30 μ M fasudil, before addition of the EC₅₀ concentration of phenylephrine (30 μ M) or 5-HT (10 μ M) (this concentration is termed **low dose** in the chapter). After 5 minutes, the tissue strips were washed with fresh warm (37°C) Krebs solution and following a 10-minute washout period, the pre-determined dose that produced a maximal contraction response of phenylephrine (300 μ M) or 5-HT (100 μ M) was added and contractile response was again measured (this concentration is termed **high dose** in the chapter). The ROCK inhibitor was present throughout the experiment. Each ureteral tissue strip was exposed to maximum of one antagonist and one agonist each. The contractile responses were measured as area under the curve and frequency.

For the second part of the study, a ROCK activity assay (Cell BioLabs, Inc) was performed on isolated distal ureteral tissue strips that were freshly homogenised with a RIPA lysis buffer system (Santa Cruz Biotechnology, Inc). The assay kit is an enzyme immunoassay developed for detection of specific phosphorylation of MYPT1 at Thr⁶⁹⁶ by ROCK. The plate provided with the kit is pre-coated with a recombinant MYPT1. The substrate wells are incubated with active ROCK standards or tissue lysate, and the phosphorylated MYPT1 is detected by an anti-phospho-MYPT1 antibody which is then visualised with a substrate solution for detection with UV spectrophotometry at 450nm. A standard curve utilizing known amounts of active ROCK-II standard was produced and used to determine the amount of active ROCK-II present in tissue lysate samples (Fig 4.1).

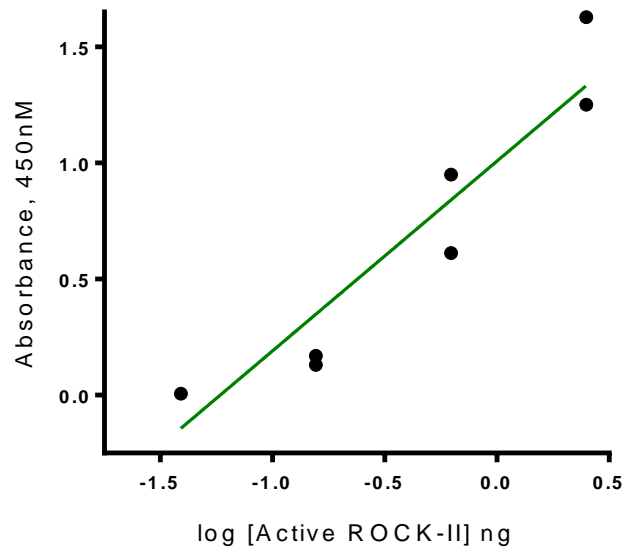


Figure 4.1 Representative standard linear regression of known amounts of active ROCK-II standard measured at 450nm.

The assay was performed by strictly following manufacturer's instructions (see Appendix 2) and using tissue lysates if isolated ureter from the distal region of both age groups: young and old, in the presence and absence of low or high doses of phenylephrine or 5-HT (similar concentrations to functional experiment). Samples were analysed in duplicates.

Paired student's *t*-tests were performed on studies with two groups while two-way ANOVA was used for studies involving more than two groups.

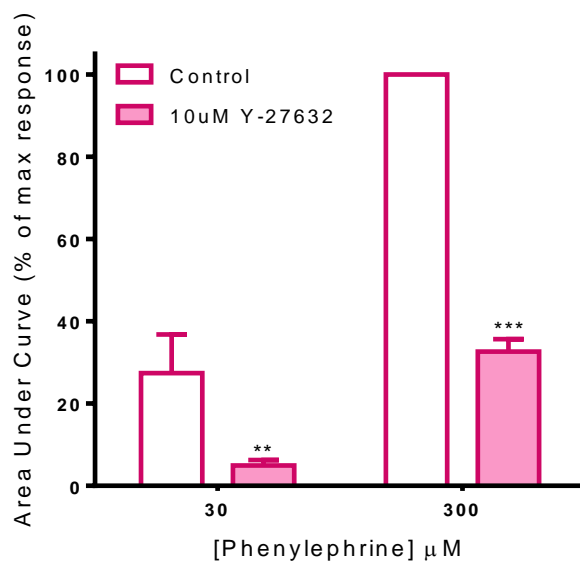
4.3 Results

All porcine ureteral strips were allowed to equilibrate to a passive tension of 1.19 ± 0.06 (n=72). Spontaneous contractions were developed during the equilibration period in 8 of 74 ureteral preparations (10.8%). When subjected to phenylephrine and 5-HT (both low and high doses), all ureteral strips from both age groups triggered bursts of phasic contractions in tissue strips. The frequency of phasic activity was increased upon addition of the high dose of phenylephrine (300 μ M) and 5-HT (100 μ M).

Effect of Y-27632 (10 μ M) on Phenylephrine-Induced Ureteral Responses

Pre-incubation with ROCK inhibitor Y-27632 (10 μ M) significantly depressed phenylephrine-induced contractions when responses were expressed as AUC at both low and high doses in isolated ureteral strips from both old and young animals (Fig 4.2a and 4.3a, paired *t*-test, $p < 0.005$). Inhibition levels at maximum AUC contraction were relatively enhanced in the older animals ($67.4 \pm 5.0\%$ vs $79.5 \pm 4.1\%$, young vs old, unpaired *t*-test, $p < 0.05$). The frequency of phenylephrine-induced contractions was only significantly inhibited by 10 μ M Y-27632 at high dose phenylephrine in ureteral strips from older animals (Fig 4.3b, paired *t*-test, $p < 0.001$). Thus, maximum inhibition on frequency of contractions by Y-27632 (10 μ M) was significantly greater in older animals in comparison to younger animals ($21.4 \pm 5.3\%$ vs $56 \pm 9.1\%$, young vs old, unpaired *t*-test, $p < 0.05$).

(a)



(b)

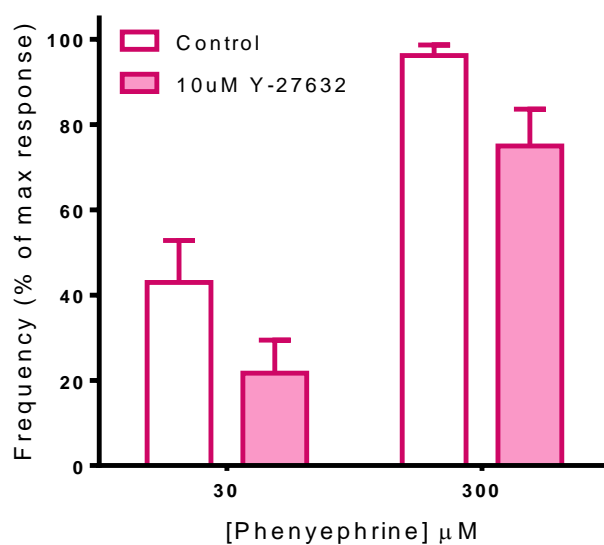
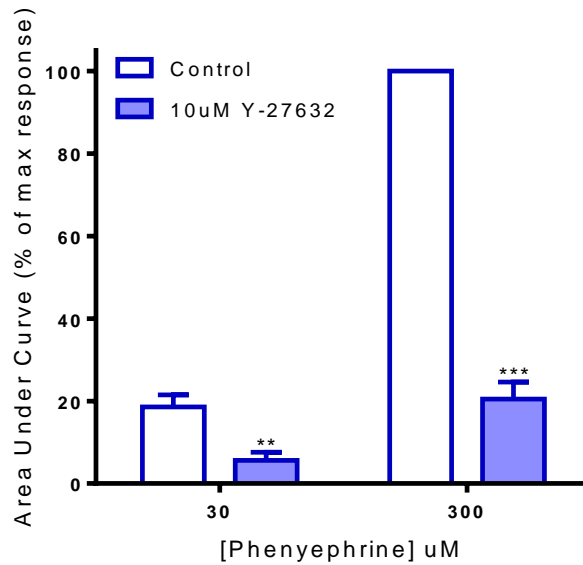


Figure 4.2 The effect of Y-27632 (10 μ M) on contractile responses to low and high doses of phenylephrine in porcine ureteral strips from young animals. Responses are represented as mean \pm SEM percentage of maximal response in AUC (a) and frequency (b) ($n = 6$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control).

(a)



(b)

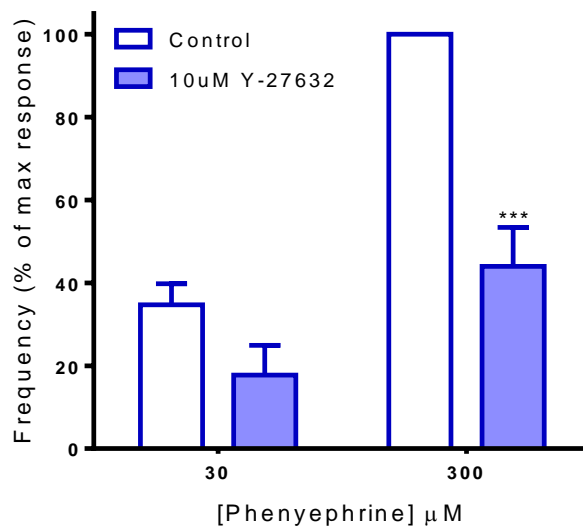


Figure 4.3 The effect of Y-27632 (10uM) on contractile responses to low and high doses of phenylephrine in porcine ureteral strips from old animals. Responses are represented as mean \pm SEM percentage of maximal response in AUC (a) and frequency (b) ($n = 8$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control).

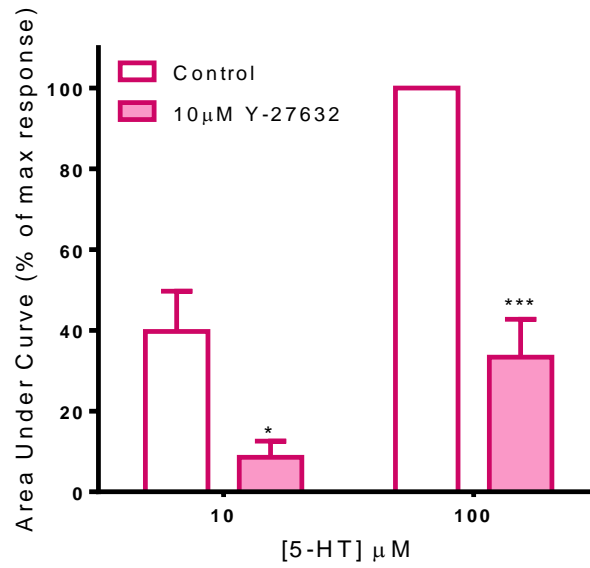
Effect of Y-27632 (10 μ M) on 5-HT-Induced Ureteral Responses

Y-27632 (10 μ M) was able to inhibit 5-HT-induced contractions at both low and high doses in both age groups (Figs 4.4a and 4.5a, paired *t*-test, $p < 0.05$). Similar to phenylephrine-induced contractions, maximal inhibition by 10 μ M Y-27632 of 5-HT contractions expressed as AUC was greater in tissues from the older animals ($66.6 \pm 7.1\%$ vs $82.6 \pm 4.8\%$, young vs old, unpaired *t*-test, $p < 0.05$). The frequency of 5-HT-stimulated contractile responses to both low and high doses was significantly inhibited by Y-27632 (10 μ M) in older animals (Fig 4.5b, paired *t*-test, $p < 0.001$) but only at the high dose in tissues from the younger animals (Fig 4.4b, paired *t*-test, $p < 0.05$). Y-27632 (10 μ M) had no effect on frequency responses to low dose of 5-HT (Fig 4.4b, $p > 0.05$). Similar to contractions expressed as AUC, maximal inhibition by the ROCK inhibitor on contraction frequency was also significantly enhanced in tissues from the older animals compared to the younger animals ($46.9 \pm 7.1\%$ vs $63.4 \pm 4.3\%$, old vs young, unpaired *t*-test, $p < 0.05$).

Comparison of Y-27632 (10 μ M) Effect on Phenylephrine- and 5-HT-Induced Ureteral Responses

When comparing the effects of Y-27632 on the two different agonists, phenylephrine and 5-HT, inhibition levels were generally similar and the only difference was that inhibition of frequency in tissues from the younger animals was greater upon stimulation by low dose 5-HT in comparison to low dose phenylephrine ($21.4 \pm 5.2\%$ vs $46.9 \pm 7.2\%$, phenylephrine vs 5-HT, unpaired *t*-test, $p < 0.05$).

(a)



(b)

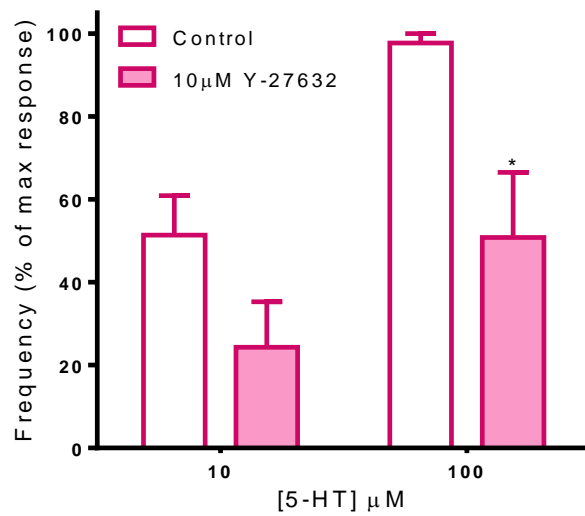
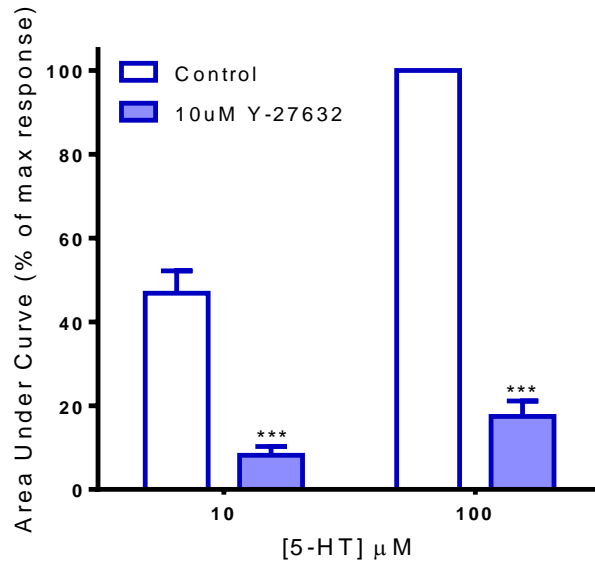


Figure 4.4 The effect of Y-27632 (10 μ M) on contractile responses to low and high doses of 5-HT in porcine ureteral strips from young animals. Responses are represented as mean \pm SEM percentage of maximal response in AUC (a) and frequency (b) ($n = 6$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control).

(a)



(b)

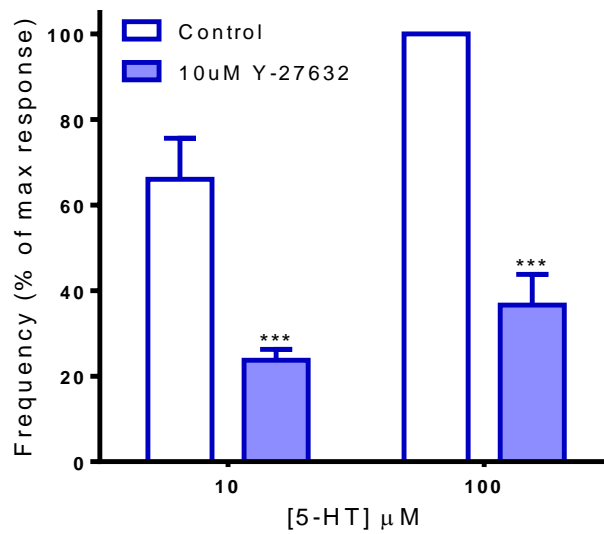
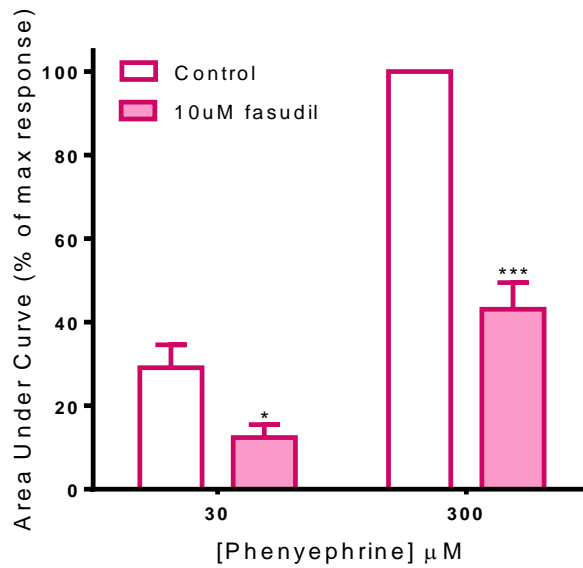


Figure 4.5 The effect of **Y-27632 (10μM)** on contractile responses to low and high doses of **5-HT** in porcine ureteral strips from old animals. Responses are represented as mean \pm SEM percentage of maximal response in AUC (a) and frequency (b) ($n = 6$, $*p < 0.05$, $**p < 0.01$, $p^* < 0.001$ vs control).

Effect of Fasudil (10 μ M) on Phenylephrine-Induced Ureteral Responses

Pre-incubation with the ROCK inhibitor fasudil (10 μ M) significantly reduced contractions to high dose of phenylephrine expressed as AUC in isolated ureteral strip from both age groups (Figs 4.6a and 4.7a, d, paired *t*-test, $p < 0.05$) but only in tissues from the younger animals at the low dose (Fig 4.6a, paired *t*-test, $p < 0.05$). At the low dose of phenylephrine, contractile responses expressed as AUC in the isolated ureter from older animals were not significantly altered in the presence of fasudil (10 μ M) (Fig 4.7a, $p > 0.05$). Inhibition by 10 μ M fasudil at maximal phenylephrine-induced contractile responses was similar in both age groups ($56.9 \pm 6.2\%$ vs $62.6 \pm 5.3\%$, young vs old, $p > 0.05$). Also, fasudil (10 μ M) had no effect on the frequency of phenylephrine-induced contractions in tissues from the younger animals (Fig 4.6b, $p > 0.05$) while in the older animals, only frequency at the high dose of 5-HT was inhibited (Fig 4.7b, paired *t*-test, $p < 0.005$). Accordingly, inhibition of max frequency responses by fasudil (10 μ M) was greater in tissues from older animals ($6.2 \pm 6.2\%$ vs $28.1 \pm 4.4\%$, young vs old, unpaired *t*-test, $p < 0.05$).

(a)



(b)

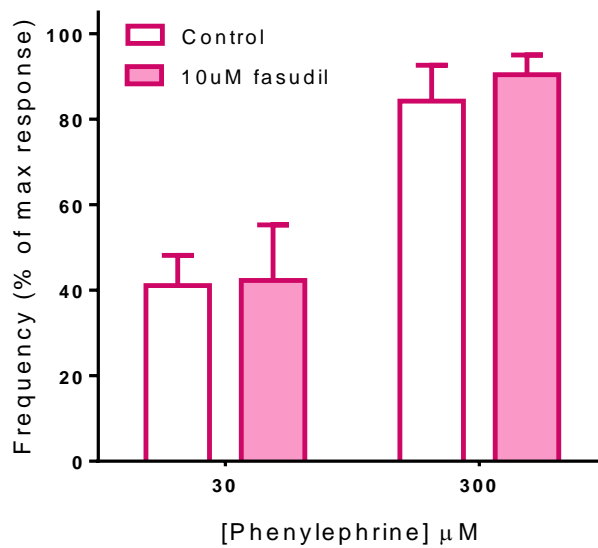


Figure 4.6 The effect of **fasudil (10μM)** on contractile responses to low and high doses of **phenylephrine** in porcine ureteral strips from young animals. Responses are represented as mean \pm SEM percentage of maximal response in AUC (a) and frequency (b) ($n = 6$, * $p < 0.05$, ** $p < 0.01$, $p^* < 0.001$ vs control).

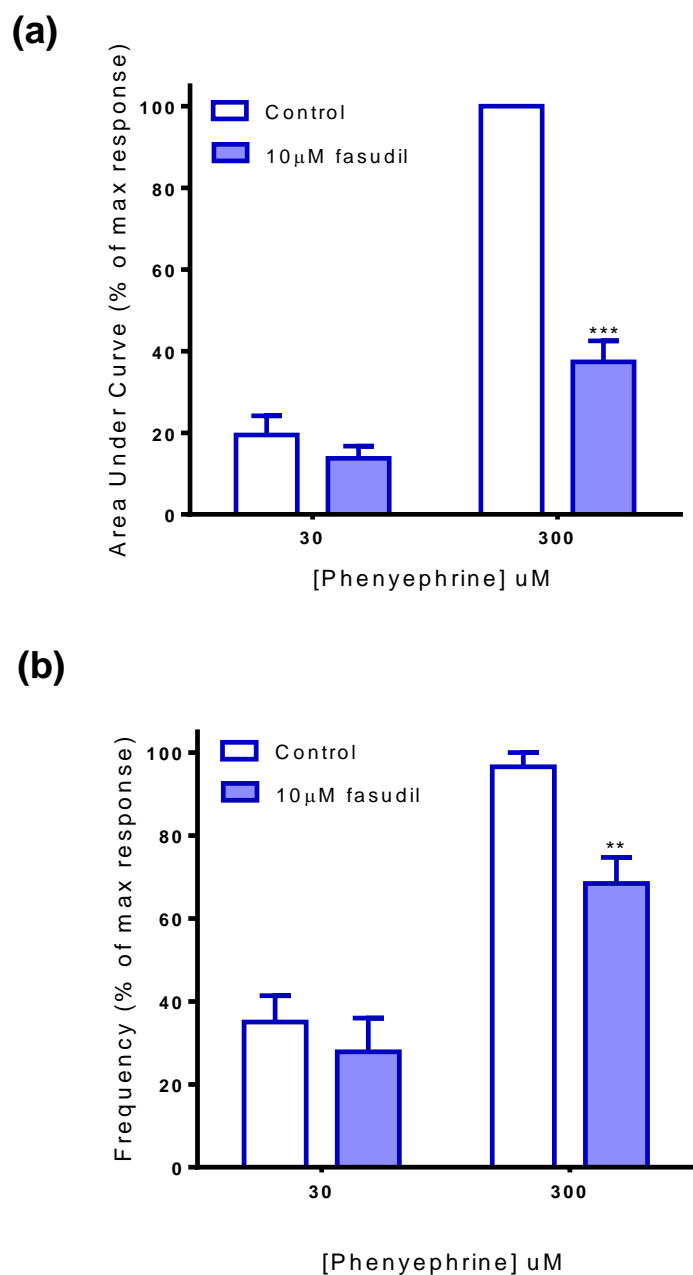


Figure 4.7 The effect of **fasudil (10 μ M)** on contractile responses to low and high doses of **phenylephrine** in porcine ureteral strips from old animals. Responses are represented as mean \pm SEM percentage of maximal response in AUC (a) and frequency (b) ($n = 6$, * $p < 0.05$, ** $p < 0.01$, $p^* < 0.001$ vs control).

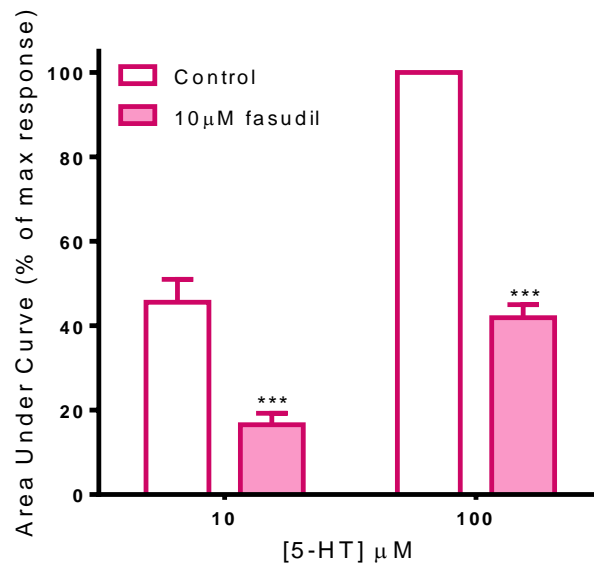
Effect of Fasudil (10 μ M) on 5-HT-Induced Ureteral Responses

Fasudil (10 μ M) depressed contractile responses to low and high dose of 5-HT when expressed as AUC in ureteral strips from both old and young animals (Fig 4.8a and 4.9a paired *t*-test, $p < 0.05$) and inhibition at high dose 5-HT of AUC contraction was similar in both age groups ($58.1 \pm 2.4\%$ vs $55.7 \pm 4.6\%$, young vs old, $p > 0.05$). The frequency of 5-HT-induced contractions was also significantly inhibited by fasudil (10 μ M) (Fig 4.8b and 4.9b, paired *t*-test, $p < 0.05$) except for responses to low dose 5-HT in tissues from the older animals (Fig 4.9b, $p > 0.05$). Inhibition levels at high dose 5-HT on frequency of contractions were also similar between age groups ($44.0 \pm 5.6\%$ vs $33.7 \pm 6.5\%$, young vs old, $p > 0.05$).

Comparison of Fasudil (10 μ M) Effect on Phenylephrine- and 5-HT-Induced Ureteral Responses

When comparing the effects of the presence of fasudil (10 μ M) on the two agonists, phenylephrine and 5-HT, inhibition levels were similar. The only exception was similar to that observed with Y-27632 (10 μ M) where frequency responses to low dose 5-HT were inhibited more than responses to low dose phenylephrine in tissues from the young animals ($6.2 \pm 6.2\%$ vs $44.0 \pm 56\%$, phenylephrine vs 5-HT, unpaired *t*-test, $p < 0.05$).

(a)



(b)

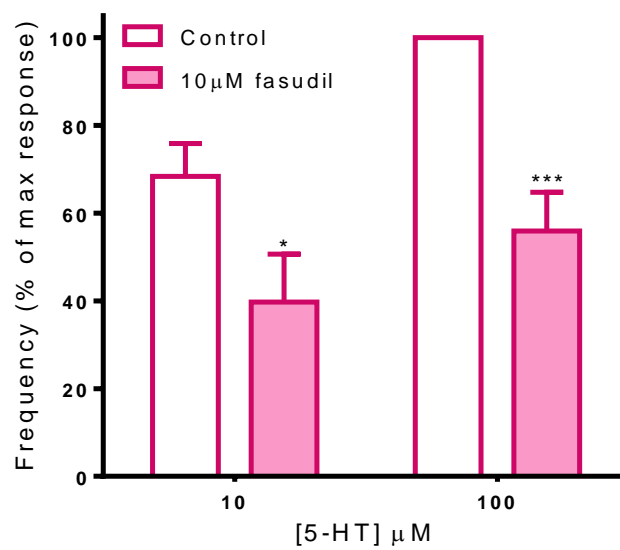
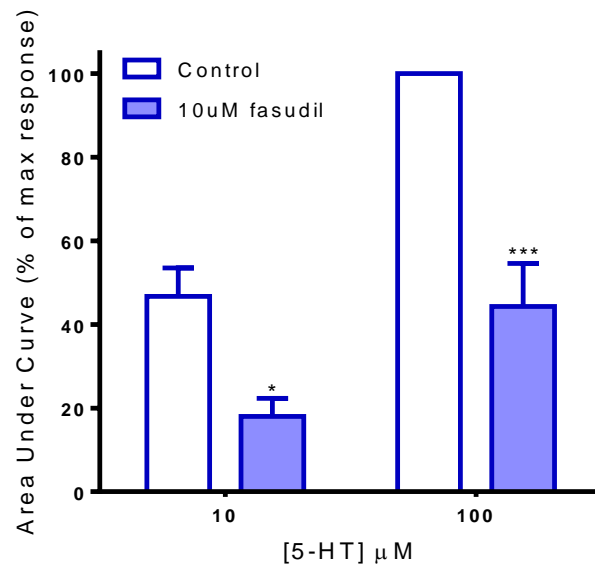


Figure 4.8 The effect of **fasudil (10 μ M)** on contractile responses to low and high doses of **5-HT** in porcine ureteral strips from young animals. Responses are represented as mean \pm SEM percentage of maximal response in AUC (a) and frequency (b) ($n = 6$, * $p < 0.05$, ** $p < 0.01$, $p^* < 0.001$ vs control).

(a)



(b)

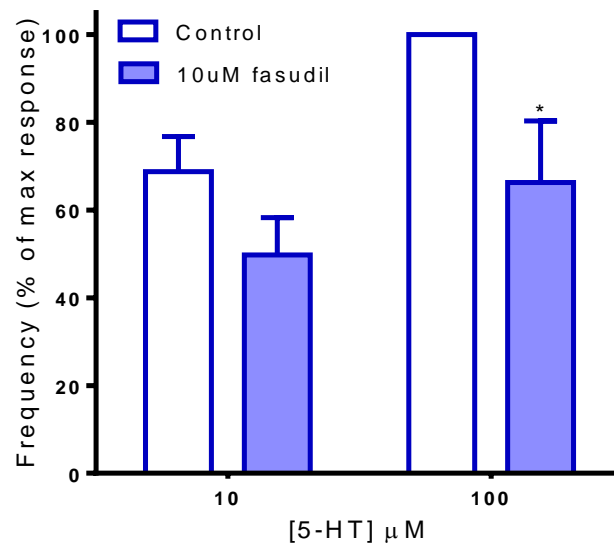
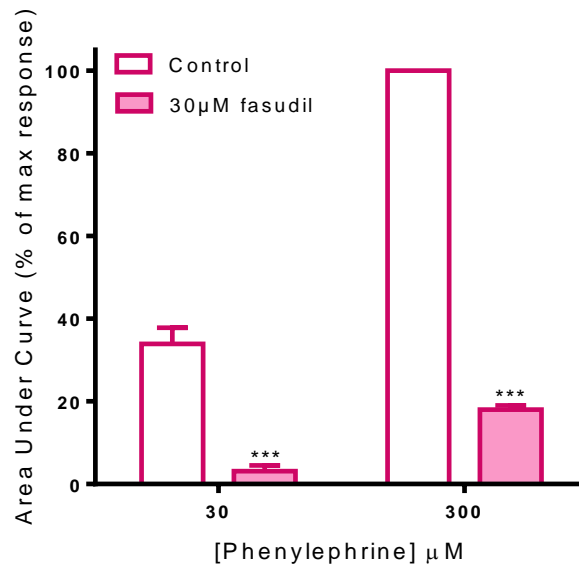


Figure 4.9 The effect of fasudil (10μM) on contractile responses to low and high doses of 5-HT in porcine ureteral strips from old animals. Responses are represented as mean \pm SEM percentage of maximal response in AUC (a) and frequency (b) ($n = 6$, * $p < 0.05$, ** $p < 0.01$, $p^* < 0.001$ vs control).

Effect of Fasudil (30 μ M) on Phenylephrine-Induced Ureteral Responses

The functional experiments in this section were then performed with a higher concentration of the second ROCK inhibitor, fasudil (30 μ M). Pre-incubation with fasudil (30 μ M) significantly reduced contractions to high and low dose phenylephrine expressed as AUC in isolated ureteral strips from both age groups (Figs 4.10a and 4.11a, paired *t*-test, $p < 0.001$) at both low and high doses of phenylephrine. Inhibition of contractile responses by fasudil (30 μ M) at high dose phenylephrine were similar in tissues from both age groups ($81.9 \pm 3.0\%$ vs $78.7 \pm 3.2\%$, young vs old, $p > 0.05$). It was also observed that fasudil (30 μ M) was effective in inhibiting frequency of phenylephrine-induced contractions at high and low doses in tissues from both older and younger animals (Figs 4.10b and 4.11b, paired *t*-test, $p < 0.05$). Additionally, inhibition of frequency responses to high dose phenylephrine was similar in both age groups ($40.9 \pm 5.2\%$ vs $29.9 \pm 4.84\%$, young vs old, $p > 0.05$).

(a)



(b)

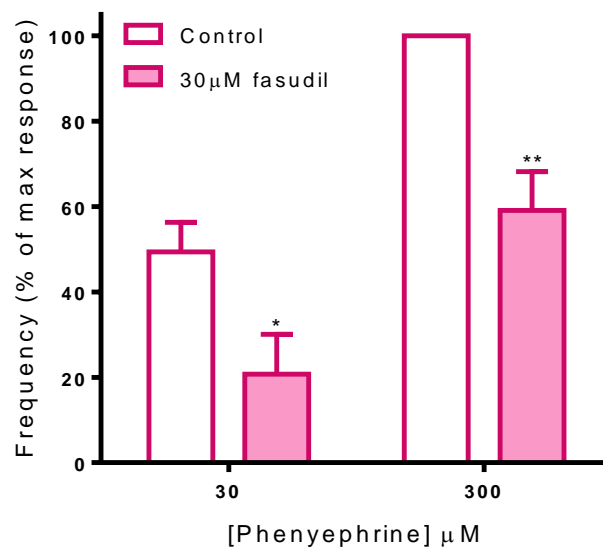
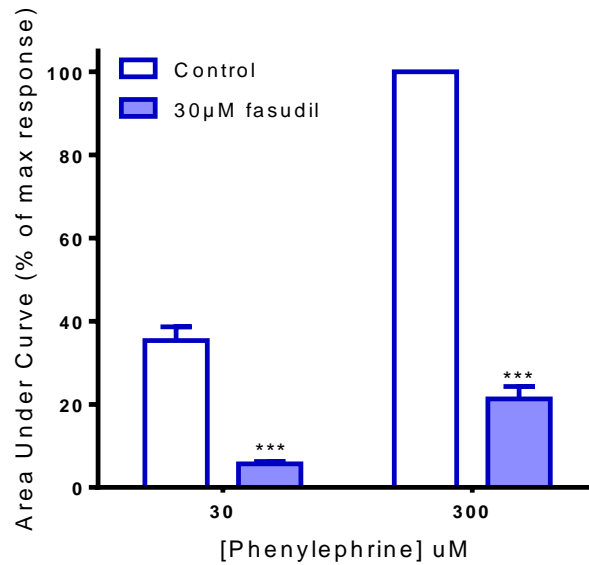


Figure 4.10 The effect of **fasudil (30 μ M)** on contractile responses to low and high doses of **phenylephrine** in porcine ureteral strips from young animals. Responses are represented as mean \pm SEM percentage of maximal response in AUC (a) and frequency (b) ($n = 6$, * $p < 0.05$, ** $p < 0.01$, $p^* < 0.001$ vs control).

(a)



(b)

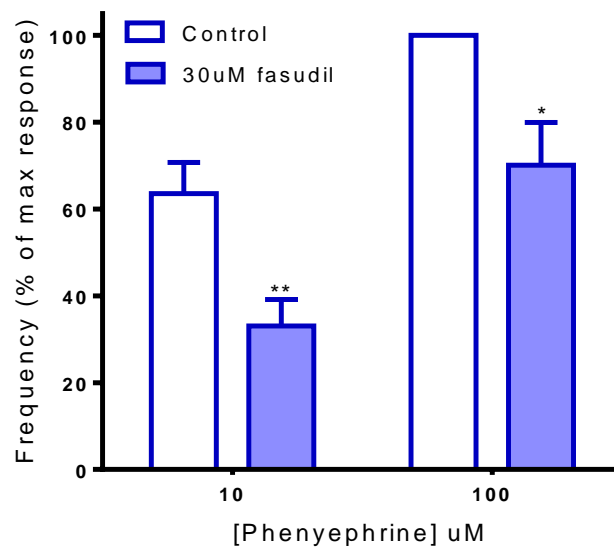


Figure 4.11 The effect of ***fasudil (30µM)*** on contractile responses to low and high doses of ***phenylephrine*** in porcine ureteral strips from old animals. Responses are represented as mean \pm SEM percentage of maximal response in AUC (a) and frequency (b) ($n = 6$, $*p < 0.05$, $**p < 0.01$, $p^* < 0.001$ vs control).

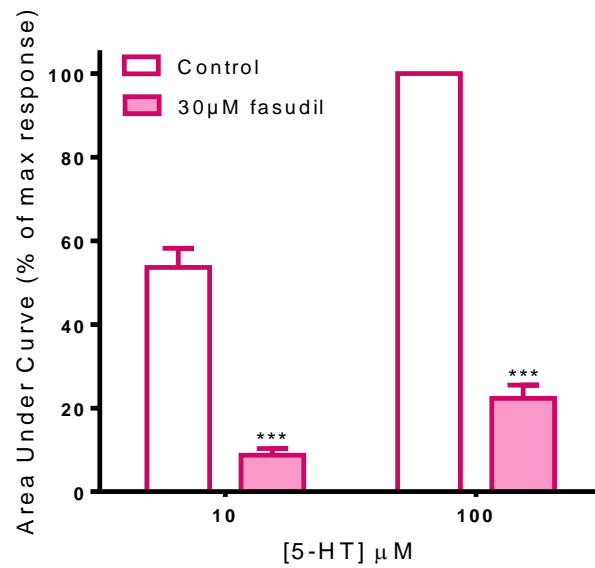
Effect of Fasudil (30 μ M) on 5-HT-Induced Ureteral Responses

Fasudil (30 μ M) suppressed contractile responses to low and high dose of 5-HT expressed as AUC in ureteral strips from both old and young animals (Figs 4.12a and 4.13b, paired *t*-test, $p < 0.001$). Inhibition of AUC contraction by fasudil (30 μ M) at high dose 5-HT was similar in both age groups ($77.1 \pm 4.1\%$ vs $79.3 \pm 2.6\%$, young vs old, $p > 0.05$). The frequency of 5-HT-induced contractions was also significantly inhibited by fasudil (30 μ M) (Figs 4.12b and 4.13b, paired *t*-test, $p < 0.005$). The degree of inhibition on high dose 5-HT-stimulated frequency responses was significantly greater in tissues from older animals in comparison to younger animals ($45.6 \pm 5.3\%$ vs $65.5 \pm 4.3\%$, young vs old, unpaired *t*-test, $p < 0.05$).

Comparison of Fasudil (30 μ M) Effect on Phenylephrine- and 5-HT-Induced contractions

When comparing the effects of pre-incubation with fasudil (30 μ M) on the two agonists, phenylephrine and 5-HT, inhibition levels were mostly similar. The only exception was that in tissues from the older animals, frequency contractile responses stimulated by high dose 5-HT was inhibited more than frequency responses to high dose phenylephrine ($29.9 \pm 4.8\%$ vs $65.5 \pm 4.3\%$, phenylephrine vs 5-HT, unpaired *t*-test, $p < 0.05$).

(a)



(b)

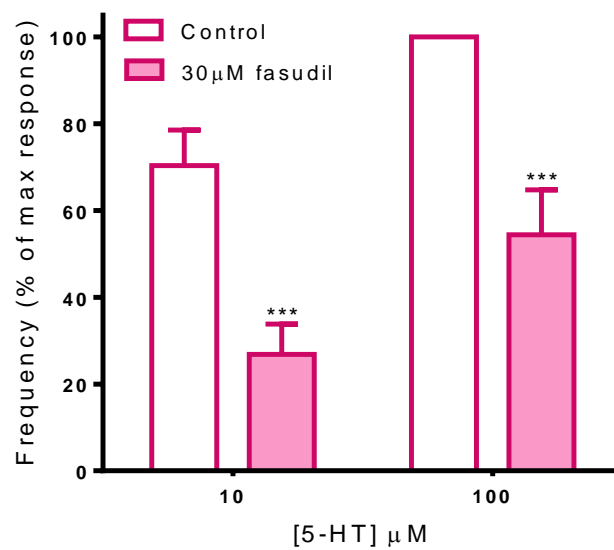
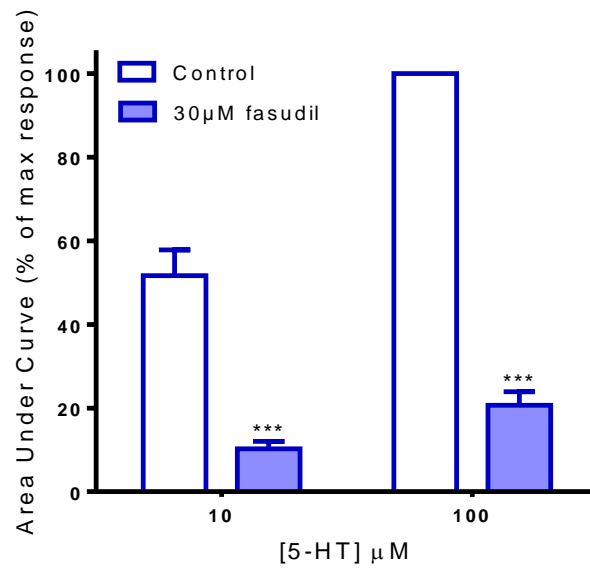


Figure 4.12 The effect of fasudil (30µM) on contractile responses to low and high doses of 5-HT in porcine ureteral strips from young animals. Responses are represented as mean \pm SEM percentage of maximal response in AUC (a) and frequency (b) ($n = 6$, * $p < 0.05$, ** $p < 0.01$, $p^* < 0.001$ vs control).

(a)



(b)

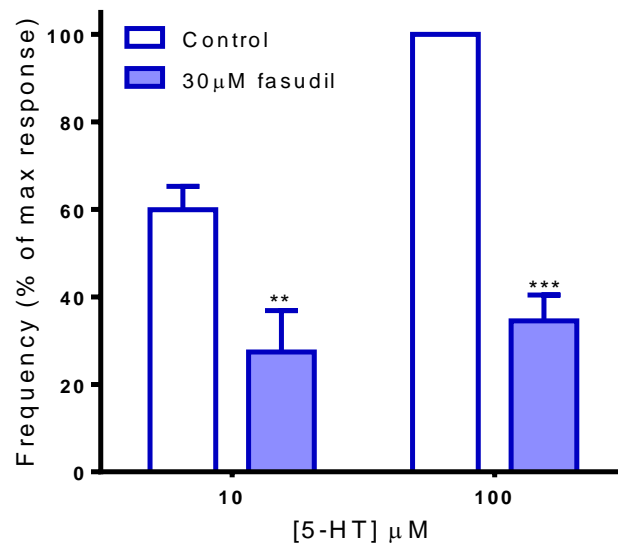


Figure 4.13 The effect of fasudil (30µM) on contractile responses to low and high doses of 5-HT in porcine ureteral strips from old animals. Responses are represented as mean \pm SEM percentage of maximal response in AUC (a) and frequency (b) ($n = 6$, * $p < 0.05$, ** $p < 0.01$, $p^* < 0.001$ vs control).

Comparison of ROCK Inhibitor Potencies on Phenylephrine-Induced Responses

The inhibitory effects on contractile responses to high dose phenylephrine by the ROCK inhibitors Y-27632 (10 μ M) and fasudil (10 μ M) were similar across all measurements (AUC and frequency) in isolated ureteral strips from the younger animals (Fig 4.14, $p>0.05$). While this was also observed in contractions expressed as AUC in tissues from the older animals, inhibition by fasudil (10 μ M) on contractile response to both agonists expressed as frequency was significantly lower than inhibition by Y-27632 (10 μ M) (Fig 4.14, two-way ANOVA, $p<0.005$).

Upon increasing the concentration of fasudil (30 μ M), inhibition of frequency responses was still significantly greater with Y-27632 (10 μ M) in tissues from the older animals (Fig 4.14, two-way ANOVA, $p<0.005$). Besides that, the inhibitory effect on contractile responses expressed as AUC in tissues from older animals was not significantly changed in the presence of fasudil at the higher concentration (30 μ M) in comparison to the lower concentration of fasudil (10 μ M). However, in the tissues from the younger animals, inhibition by fasudil (30 μ M) on phenylephrine-induced contractions expressed as AUC and frequency were both significantly increased in comparison to the lower concentration of fasudil (10 μ M) (Fig 4.14, two-way ANOVA, $p<0.05$). In this age group also, inhibition of contraction frequency by fasudil (30 μ M) was enhanced to the point where it was significantly greater than inhibition by 10 μ M Y-27632 (Fig 4.14, two-way ANOVA, $p<0.05$).

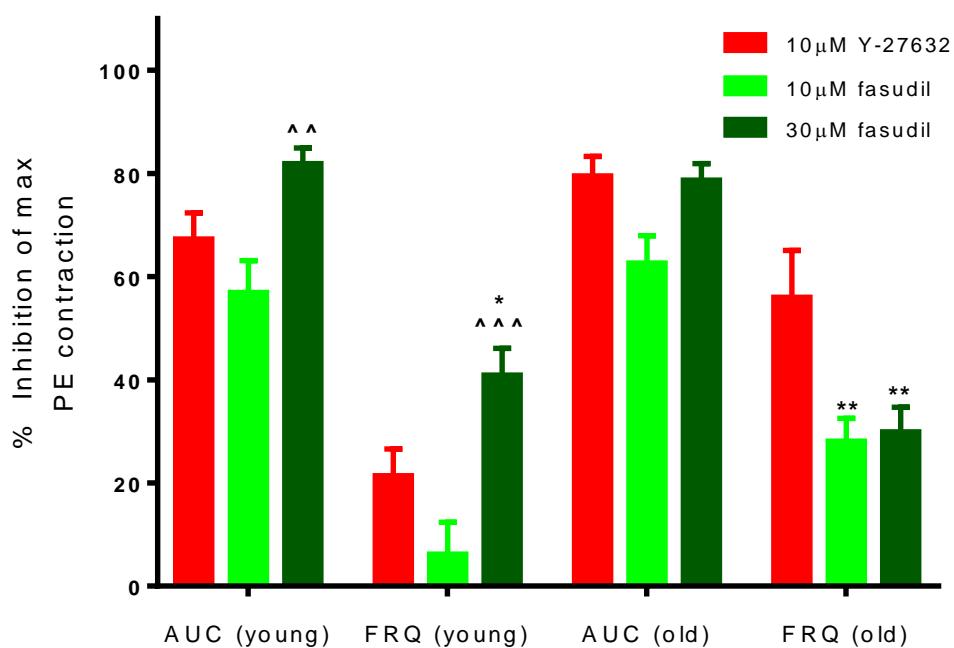


Figure 4.14 Percentage inhibition of phenylephrine-induced (300μM) contractile responses by ROCK inhibitors, Y-27632 (10μM) and fasudil (10μM and 30μM) expressed as AUC and frequency. Responses are represented as a mean percentage inhibition \pm SEM ($n=6$, * $p<0.05$ vs 10μM Y-27632, ^ $p<0.05$ vs 10μM fasudil).

Comparison of ROCK Inhibitor Potencies on 5-HT-Induced Responses

The inhibitory effects on contractile responses to high dose 5-HT by the ROCK inhibitors Y-27632 (10 μ M) and fasudil (10 μ M) were similar across all measurements (AUC and frequency) in isolated ureteral strips from the younger animals (Fig 4.15, $p>0.05$). In contrast, these similarities were not observed in tissues from older animals as inhibition of responses by fasudil (10 μ M) expressed as both AUC and frequency were significantly lesser in comparison to Y-27632 (10 μ M) (Fig 4.15, two-way ANOVA, $p<0.005$).

Upon increasing the concentration of fasudil (30 μ M), inhibition of AUC and frequency responses in the older animals were significantly greater than the lower concentration (10 μ M) (Fig 4.15, two-way ANOVA, $p<0.005$). Additionally, the inhibition at the higher concentration of fasudil (30 μ M) was similar to Y-27632 (10 μ M) (Fig 4.5, $p>0.05$). In tissues from the younger animals, the inhibitory effect of responses expressed as AUC was increased by the higher concentration of fasudil (30 μ M) in comparison to the lower concentration (10 μ M) (Fig 14.5, two-way ANOVA, $p<0.05$). However, this increase was not observed in responses expressed as AUC (Fig 14.5, $p>0.05$). Also, inhibition of both AUC and frequency responses by fasudil (30 μ M) was indifferent to inhibition by Y-27632 (10 μ M) in the younger group (Fig 14.5, $p>0.05$).

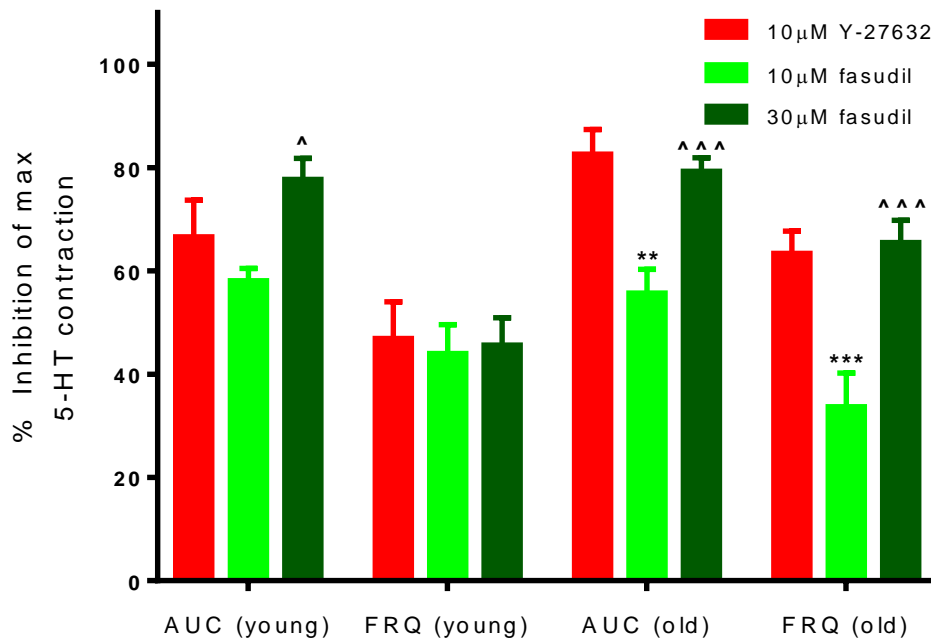


Figure 4.15 Percentage inhibition of 5-HT-induced contractile responses (100μM) by ROCK inhibitors, Y-27632 (10μM) and fasudil (10μM and 30μM) expressed as AUC and frequency. Responses are represented as a mean percentage inhibition \pm SEM (n=6, * p <0.05 vs 10μM Y-27632, [^] p <0.05 vs 10μM fasudil).

Rho-kinase Activity

Using the standard curve for ROCK-II sample (Fig 4.1), active ROCK levels in samples of tissue lysates from different groups was determined. As seen in Figure 4.16, basal levels of active ROCK were found to be similar in old and young groups and were not elevated following agonist stimulation with either phenylephrine or 5-HT. The only changes in ROCK activity that were observed between tissues from old and young animals with greater ROCK activity in tissues from old group at high dose 5-HT (100 μ M) and lower levels in tissues from younger animals after low dose of phenylephrine (30 μ M) (Fig 4.16, two-way ANOVA, $p < 0.05$).

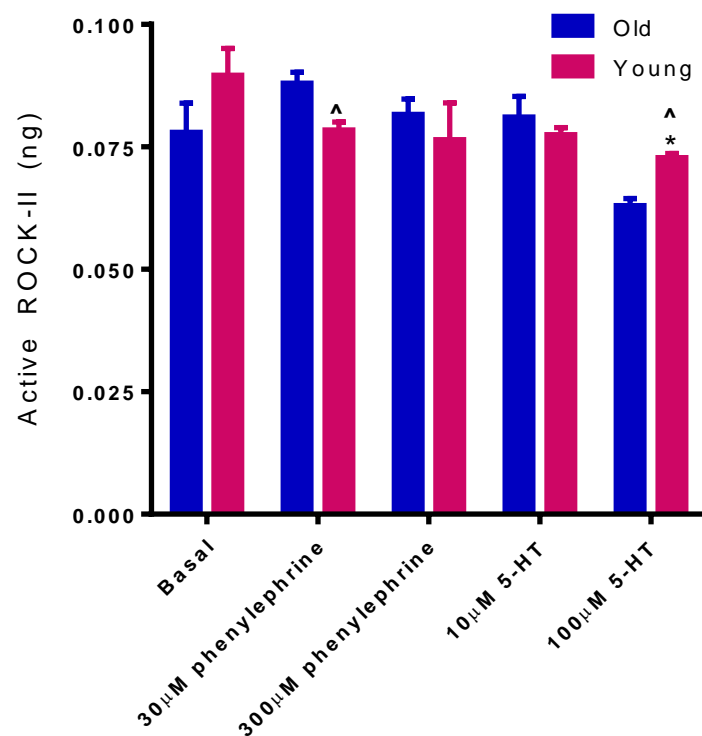


Figure 4.16 Active rho-kinase levels at basal, low and high doses of phenylephrine- and 5HT-induced states in isolated porcine ureteral smooth muscle in old and young animals. Responses are represented as mean \pm SEM (* $p < 0.05$ vs basal, ^ $p < 0.05$ vs old) of 3 determinations, each performed in duplicate.

4.4 Discussion

Effect of Rock Inhibitors on Agonist-Induced Contractions

The present study sought to investigate the importance of the ROCK pathway in agonists-induced contractile responses of the distal ureter. The results demonstrate the ability of phenylephrine and 5-HT to activate the ROCK pathway in the ureter and therefore, confirmed previous reports suggesting a role for of the calcium sensitization pathway in ureteral contractions (Levent and Buyukafsar, 2004, Shabir et al., 2004, Shabir et al., 2013).

The two agonist concentrations that were chosen for the organ bath experiments were determined based on previous concentration-response curves produced in respective age groups for the two agonists. The low dose represents EC_{50} where contractile responses were 50% of its maximum response, while the high dose was the concentration producing the maximal contractile response (100%).

The main finding in our functional study was that both ROCK inhibitors Y-27632 and fasudil were able to suppress phenylephrine- and 5-HT-induced contractions in the porcine distal ureter. Involvement of ROCK activation in α_1 -adrenoceptor-dependent ureteral stimulation has been characterised in sheep ureter (Levent and Buyukafsar, 2004). Based on our current knowledge of the literature, we are the first to demonstrate the activation of ROCK in response to 5-HT stimulation. Therefore, based on our functional studies, we confirmed that ROCK significantly contributes to phenylephrine- and 5-HT-stimulated contractile responses in the porcine distal ureter.

In addition to these major findings, interestingly, we also found that there were differences in inhibition induced by the two ROCK inhibitors, Y-27632

and fasudil at similar concentration in the different age groups. We demonstrated that the inhibition of agonist response by Y-27632 (10 μ M) was more effective for than fasudil (10 μ M) for both phenylephrine and 5-HT-induced contractions, but only in ureters from older animals and not in those from young pigs. This decreased degree of inhibition with fasudil (10 μ M) for phenylephrine was only observed in frequency response and not AUC responses. This raises the question as to whether there are different pathways involved in the mediation of tonic and phasic α_1 -adrenoceptor mediated contraction in tissues from the older animals.

Increasing the dose of fasudil from 10 μ M to 30 μ M (3-fold) produced an increase in inhibition equivalent to the level produced by Y-27632 (10 μ M), but this was only apparent in frequency of 5-HT-stimulated concentrations and not in phenylephrine-induced responses. Also, upon increasing the fasudil concentration (30 μ M), the inhibitory effect was significantly increased in the ureters from younger animals for both phenylephrine and 5-HT, indicating that there could be potential differences in efficacy of each ROCK inhibitor for different age groups. To further prove this point, we also observed that the inhibitory effects of Y-27632 (10 μ M) on contractile responses expressed as AUC and frequency to both agonists were significantly greater in isolated ureter from older animals in comparison to the younger animals.

In a study of the human detrusor, it was found that inhibitory effects by fasudil and Y-27632 on carbachol-induced contractions were dose-dependent and comparison between contractions in increasing doses was performed (Kirschstein et al., 2014). Similar to our study, this study reports a greater inhibitory effect of Y-27632 on carbachol-induced contractions in comparison to fasudil (Kirschstein et al., 2014). This could be explained by the possibility that Y-27632 is not as specific for ROCK as we expected as it can directly inhibit phosphorylation of CPI-17 by purified PKC δ at the same concentration required to inhibit ROCK (Eto et al., 2001). This

possible additional action of Y-27632 also results in suppression of smooth muscle contraction (Li et al., 1998). Because inhibition of phenylephrine-stimulated contraction frequency was greater by Y-27632 than fasudil (both low and high concentrations) in the older animals, one suggestion would be that this specific agonist-mediated contraction is mediated by both ROCK and CPI-17 inhibition. However, at the same time, ROCK has also been proposed to have the capability of phosphorylating CPI-17 (Li et al., 1998). Thus, it is difficult to verify if inhibition of CPI-17 was due to ROCK pathway inhibition or due to the action of Y-27632 and further investigation is still required to accurately confirm whether CPI-17 phosphorylation is involved in modulation of ureteral smooth muscle contractions. Nevertheless, it is undeniable that our results indicate that the effectiveness of ROCK inhibitors might be age-related and also agonist-related in older animals which proves to be more complex than anticipated.

Basal and Agonist-Induced Rho-kinase Activity

Our findings show that there was an overall dissimilarity between basal ROCK activity and agonist-induced activity in tissue lysate of distal ureter from both age groups (only significantly lower in 100 μ M 5-HT in tissue lysate from young animals). Upon addition of ROCK inhibitors to tissue strips, there was no decrease in baseline tone, but this does not necessarily indicate that there was no ROCK activity present at basal levels since baseline was set to a very low tension (approximately 1g).

Regrettably, we did not have sufficient numbers of tissue strips for each group (control and inhibitor) that developed spontaneous contractions to statistically determine if ROCK inhibitors had inhibitory effects on basal frequency responses. However, several studies have shown that ROCK inhibitors are capable of inhibiting spontaneous contractions of the ureter in various species including rat (Shabir et al., 2004), sheep (Levent and Buyukafsar, 2004), and human (Hong et al., 2005). These studies suggest

that ROCK might be activated without the stimulation of agonists, supporting our assay results.

Several studies on the bladder have demonstrated that ROCK expression and ROCK activity might increase with age (Gomez-Pinilla et al., 2011, Kirschstein et al., 2014). In younger animals in comparison to older animals, we found ROCK activity was lower for low dose phenylephrine (30 μ M). The opposite was observed with high dose 5-HT (100 μ M) where younger animals had greater ROCK activity. While we acknowledge that conclusions cannot be drawn as these alterations are only observed at one concentration and not the other, these findings suggest again, that alterations of ROCK activity occur with ageing.

Interestingly, our previous data on the effects of age on agonist-stimulated contractions (Chapter 2) demonstrated an increase in contractile responses to phenylephrine and carbachol in older animals and greater 5-HT-stimulated contractions in younger animals. In addition to this, it has been shown in the human bladder that mediation of carbachol-induced contractions via the ROCK pathway is positively correlated with age (Kirschstein et al., 2014). Our findings indicate that changes in the role of the ROCK pathway with age could be dependent on the agonist being studied.

4.5 Conclusion

Currently, nifedipine, a calcium channel blocker is one of the drugs clinically used to treat ureteral calculi (Osorio et al., 2008). However, nifedipine has been shown to result in various significant adverse effects including nausea, vomiting, dizziness and flushing (Singh et al., 2007, Osorio et al., 2008). Therefore, the discovery of the calcium sensitization pathway in smooth muscle contraction opens the window for the possibility of a new therapeutic target for drug development. Supporting results from previous ROCK studies in ureters from various species, our studies further support the view that the ROCK pathway plays a major role in normal ureteral function. There is evidence that ROCK is up-regulated in ureteral obstruction (Turna et al., 2007) and thus, it could be an interesting target for drugs aimed at increasing ureteral stone expulsion rate and reducing ureteral colic by relieving ureteral pressure. We also demonstrated that ROCK inhibitors might have altered effects with ageing. This discovery could be vital in prescribing specific treatments for specific age groups. However, it is clear that further studies are needed to further elucidate the mechanisms through which α_1 -adrenoceptor and 5-HT stimulation induces the ROCK activation. Additionally, it will also be beneficial to clarify the potential of utilising ROCK inhibitors therapeutically *in vivo* and in clinical settings as a part of medical expulsive therapy.

CHAPTER 5:

The effect of urothelium-derived
inhibitory factor and adenosine
triphosphate on ureteral contractile
responses

5.1 Introduction

The urothelium is a stratified epithelium that lines the inner parts of the renal pelvis, ureters, urinary bladder and part of the urethra. The urothelium can be divided into three layers composed of different cell types; the outermost, middle and innermost layers (Figure 5.1). The outermost layer of the urothelium is made up of umbrella cells which are large, flattened and firmly interlocked by tight junctions. The middle layer is made up of interstitial cells, while the innermost layer is composed of smaller basal cells that are separated by the basal lamina from the suburothelial lamina propria (Jost et al., 1989). Although the number of intermediate layers might vary between species, it is well known that these three layers of cells are present in most species, including human and pig (Winder et al., 2014).

Until recently, the urothelium has always been considered to function solely as a barrier against the contents of the urine. While this role still proves true, today, many dynamic qualities of the urothelium have been well recognised. It has been shown that the urothelium is able to release signalling molecules acting at different receptor subtypes within the urothelium and also able to modify afferent neuronal activity and detrusor smooth muscle function (Lazzeri, 2006, Birder and Andersson, 2013). There is limited reported research on the urothelium in the ureter. Since the vast majority of studies on the urothelium revolve around the urinary bladder, it is tempting to assume that the urothelial lining of the ureter will most likely have similar morphology and functionality, especially in the distal ureter as it is closest to the bladder.

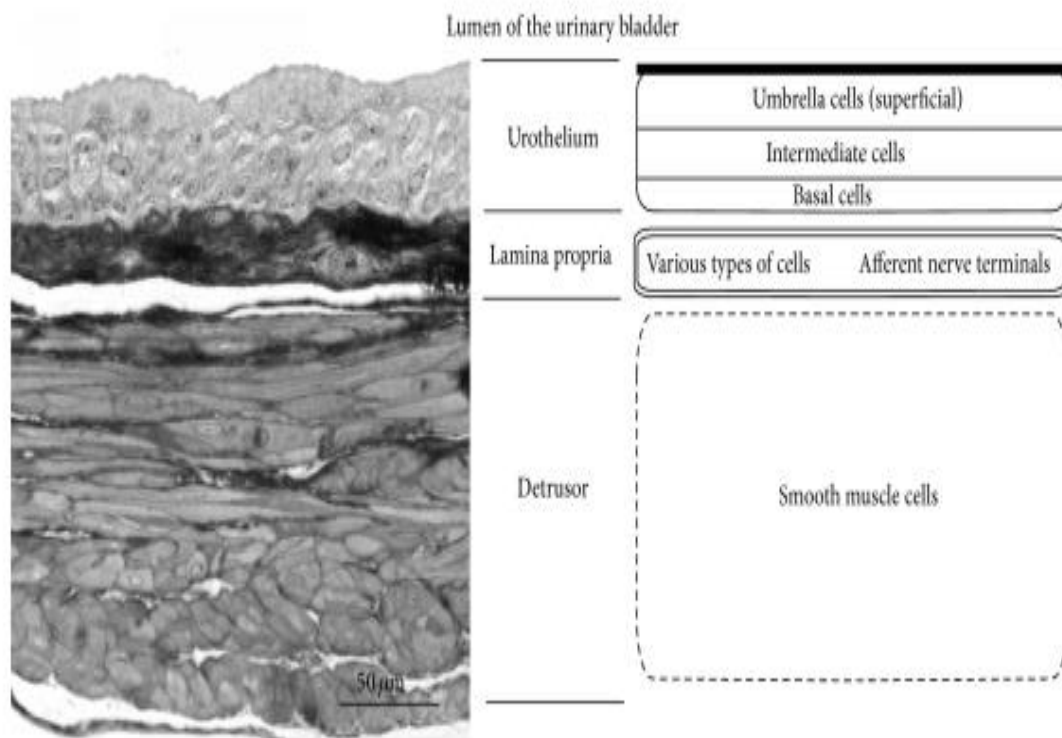


Figure 5.1 Urinary bladder wall of the mouse, including a schematic representation of the urinary bladder wall. The urothelium is composed of superficial umbrella cells, intermediate cells, and basal cells. Beneath the urothelium there is suburothelial connective tissue, which is called the lamina propria. It is composed of various types of cells including fibroblasts, interstitial cells, and myofibroblasts and also afferent nerve terminals. The outermost layer is the detrusor smooth muscle cell layer (Winder et al., 2014).

One of the most significant and novel functions of the urothelium is its ability to act as a mechanosensory conductor (Ferguson et al., 1997, Andersson, 2002). It has been shown that upon distension of the bladder wall, the stretching of the urothelium occurs, leading to the release of several signalling molecules (Olsen et al., 2011). Among these signalling

molecules are acetylcholine, NO and prostaglandins, but the one that appears to play an important role is adenosine-5'-triphosphate (ATP) and has been most characterised (Yoshida et al., 2006, Andersson and Persson, 1994).

ATP is well-known as a multifunctional ubiquitous biological molecule, acting as the main intracellular energy source for all living cells and also an extracellular signalling molecule. ATP has been predominantly associated with the activation of afferent signalling and the stimulation of purinergic receptors on afferent nerve terminals is now well-accepted as a mechanosensory mechanism (Apostolidis et al., 2005). In the urinary bladder, it has been shown that it is a signalling molecule with a vital role in filling of bladder sensation (Burnstock, 2014).

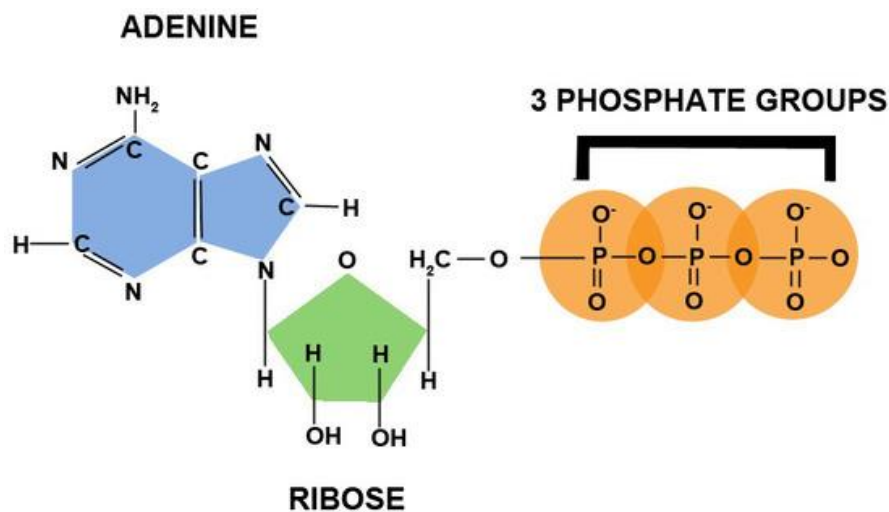


Figure 5.2 Structure of ATP. ATP consists of an adenine attached to a ribose sugar, which is attached to three phosphate groups.

The formation of ATP is an energetically unfavourable phosphorylation reaction where a phosphate group is added to adenosine diphosphate (ADP). Most ATP molecules in cells are produced in the mitochondria via oxidative phosphorylation while a small amount is produced in the cytosol via glycolysis. While ATP is abundant in the cell cytoplasm (2-5mM), there is a higher concentration of ATP (100mM) stored in synaptic vesicles of neurons (Winder et al., 2014)

It has always been assumed that extracellular ATP is only sourced in damaged or dying cells until recently. It is now apparent that ATP can be released from many different cell types including peripheral and central neurons and also many non-neuronal cell types during mechanical deformation including shear stress, stretch, hypoxia, osmotic swelling and stimulation by agents (Bodin and Burnstock, 2001, Boudreault and Grygorczyk, 2004). It was first suggested that ATP is released from epithelial cells lining structures that are viewed as 'sacs' (bladder and lungs) or 'tubes' (ureter, gut) (Burnstock, 1999).

ATP is a highly charged molecule and cannot readily cross plasma membranes, thus physiological release requires the participation of an active transport mechanism. While theories on actual transport mechanisms involved in the release of ATP are still contradictory, there is compelling evidence that in neurons, the mechanism involves exocytotic vesicles (Novak, 2003). On the other hand, in non-neuronal cells like red blood cells and epithelial cells, several other mechanisms (on top of vesicle transport) have been proposed including the ATP-binding cassette transporters (Burnstock, 2007, Demolombe and Escande, 1996) and transport via connexin or pannexin channels (Stout et al., 2002, Timoteo et al., 2014). In the urothelium, the pathways underlying release of ATP are mostly attributable to vesicular exocytosis (Wang et al., 2005) and pannexin hemichannel conductive efflux (Timoteo et al., 2014, Negoro et al., 2013) although there is still no consensus on this. Regardless of the

transport mechanism, it is agreed that the urothelium has the capability to release ATP into both mucosal and serosal compartments of the urinary bladder wall (Ferguson et al., 1997, Lewis and Lewis, 2006) and can thus, play an autocrine function (Wang et al., 2005) and/or act in a local paracrine manner to influence afferent nerves, interstitial cells and myofibroblasts or detrusor muscle contractility (Cockayne et al., 2000, Sui et al., 2014).

It is hypothesised that the initiation of ATP release from epithelial cells is the deformation of these cells (Burnstock, 1999). In the urinary bladder, the release is thought to be caused by stretching of the urothelium during the filling of the bladder. This increases the tension in the apical layer of umbrella cells and therefore an increase in apical surface area of these cells, which is suggested to result in the activation of mechanotransduction pathways, via stretch-activated release of ATP from the urothelial cells (Ferguson et al., 1997, Cheng et al., 2011, Dunning-Davies et al., 2013, Wang et al., 2005). In several animal models, it has been reported that the amount of ATP released during distension was proportional to the extent of distension (Ford and Cockayne, 2011, Vlaskovska et al., 2001). The actual cell type/s in the urothelium responsible for the release of ATP is still unidentified but it was demonstrated that the umbrella cells, intermediate and basal subtypes were equally effective in ATP generation (McLatchie and Fry, 2015).

Upon the release of ATP extracellularly, it can act as an autocrine or paracrine signal, modulating cell functions via activation of purinergic receptors located on the plasma membrane of cells. The two purinergic receptor types are P1 receptors, which are selective for adenosine, formed during the breakdown of ATP (also known as adenosine receptors) and P2 receptors, which are selective ATP and ADP (Burnstock, 2007). P2 receptors are further classified into two families: the P2X and P2Y family. Currently, there are seven known P2X receptor subtypes and eight P2Y

receptor isoforms (North, 2002, Shaver, 2001). While results from several studies regarding P2 receptor expression in the urinary bladder are conflicting, it appears that the urothelium expresses all seven P2X receptor subtypes and several P2Y receptor subtypes including P2Y₁, P2Y₂, and P2Y₄ (Khandelwal et al., 2009, Shabir et al., 2013).

Expression of both P2X and P2Y purinergic receptors are specifically located at the afferent nerve fibres and interstitial cells/myofibroblasts situated near the bladder luminal surface. The sensitivity of these cells to ATP suggests that the basolateral ATP release could also affect their functions (Birder and de Groat, 2007). P2Y receptors are thought to be involved in the mediation of further ATP release in the native bladder mucosa. UTP, a P2Y receptor selective agonist, was shown to significantly increase ATP release and in contrast, α,β -methylene ATP (α,β -meATP), a selective P2X₁ and P2X₃ agonist, had no significant release of ATP in the bladder mucosa (Sui et al., 2014).

It is suggested that in the urothelium, stimulation of P2X₂ and P2X₃ receptors present on the urothelium by ATP released from the apical and basolateral urothelial surfaces induced stretch-induced exocytosis and endocytosis (Wang et al., 2005). It is proposed that in the urothelial cells, exocytosis and endocytosis may modulate the composition of receptors present and absent at the plasma membrane of urothelial cells (Winder et al., 2014). In addition to this, it is also hypothesised that stimulation of P2X₃ receptors on suburothelial sensory nerve fibres by ATP from the basolateral surface of the urothelium during bladder filling plays an important role in the relaying of information regarding the degree of bladder filling to the central nervous system (Cockayne et al., 2000). In support of this hypothesis, it was shown in knockout mice without P2X₂, P2X₃ or P2X₂/P2X₃ receptor subunits, there is limited activation of bladder afferent nerve terminals and reduced micturition frequencies and increased bladder volume capacities (increased voiding volume) in

comparison to control mice. Despite this, in the knockout mice, ATP release from the bladder urothelium is unaltered (Cockayne et al., 2005).

Although limited research on the urothelium of ureter is available, studies on the guinea pig (Knight et al., 2002) and human (Calvert et al., 2008) ureter demonstrated an intraluminal pressure-dependent release of ATP from the urothelium. It is proposed that the transport mechanism of urothelial ATP release appears to involve vesicular exocytosis and maybe ABC transporters in the guinea pig ureter (Knight et al., 2002). Interestingly, the release of ATP only occurred at a certain intraluminal pressure threshold and this threshold was comparable to pressure threshold in ureteric model mimicking obstructing calculi with the insertion of an inflated balloon where ureteral colic is experienced (Risholm, 1954, Calvert et al., 2008).

Another interesting note is that the P2X₃ homomultimer and P2X_{2/3} heteromultimer receptor subtypes have been identified on nociceptive sensory neurons and therefore have been associated with the sensation of pain (Burnstock and Wood, 1996, Chen et al., 1995, Lewis et al., 1995). It is proposed that ATP released acting as a signal for distension of structures could act on P2X_{2/3} receptors on the subepithelial sensory nerves to convey information to the central nervous system pain centres (Burnstock, 1999). This has been exhibited in urinary bladder of various species (Ferguson et al., 1997, Vlaskovska et al., 2001) and also in the guinea pig ureter (Rong and Burnstock, 2004). This is further supported by evidence that P2X₃ receptors are present on occasional nerve bundles in the subepithelial plexus of the rat (Lee et al., 2000) and human (Calvert et al., 2008) ureter. These findings suggest a significant role of ATP where distension of ureteral wall by calculi and increased intraluminal might stimulate its release, and therefore stimulation of this nociception pathway.

With relation to this, the urothelium has been implicated in a number of lower urinary tract disorders including, cyclophosphamide-induced cystitis and interstitial cystitis/painful bladder syndrome, where increased urothelial ATP release has been shown, in addition to the altered sensitivity of afferent nerve terminals (Jost et al., 1989, Ferguson et al., 1997). There is also indication that expression of P2X₂ and P2X₃ receptors is upregulated in the bladder of patients with interstitial cystitis (Tempest et al., 2004).

In addition to disease, urothelial ATP release mechanisms have also been shown to be altered with ageing. Altered ATP and acetylcholine bioavailability and increased P2X₃ receptor expression at the urothelium was demonstrated in the aging mouse bladder (Daly et al., 2014). Furthermore, intrinsic ATP release in guinea pig mucosal preparations was also significantly enhanced in tissues from older animals (Sui et al., 2014).

Much has been discussed regarding the urothelium's role in transducing and integrating sensory signals, especially via urothelial ATP release, for the nervous system to encode for relay to the central nervous system. Besides this notable function, it is also apparent that the urothelium can affect the contractility of the underlying smooth muscle layers. In addition to chemical diffusion between the urothelium and smooth muscle layers, it is suggested that gap junctions in the myofibroblasts could also be a mechanism for long-distance spread of signals from the urothelium to the detrusor smooth muscle (Brading, 2006).

Many have shown that the removal of urothelium significantly enhances detrusor smooth muscle contractility to a range of contractile agents (Pinna et al., 1992, Maggi et al., 1987, Levin et al., 1995, Murakami et al., 2007). In the pig urinary bladder, it was demonstrated that detrusor smooth muscle contractions to carbachol were inhibited in strips denuded

of urothelium in the presence of a second strip with a functional urothelium, indicating the release of a diffusible inhibitory agent from the urothelium upon activation of muscarinic stimulation (Hawthorn et al., 2000). This was also further confirmed when these responses were potentiated following the removal of urothelium from previously intact bladder strips (Hawthorn et al., 2000). This inhibitory agent is yet to be identified and is termed the urothelium-derived inhibitory factor (UDIF). Besides muscarinic stimulation, it was suggested that β -adrenoceptor stimulation also induces the release of UDIF (Murakami et al., 2007). The presence of UDIF has also been demonstrated in the rat ureter, where stimulation by various agonists including carbachol, bradykinin, angiotensin II, neurokinin A and vasopressin were enhanced in urothelium-free tissue strips (Mastrangelo and Iselin, 2007). These findings indicate that UDIF depresses muscle contractility by either metabolism or inactivation of agonists, acting as a barrier to diffusion and penetration of agonist, or by releasing subsequent agents that reduced muscle contractility (Birder et al., 2012).

Several possible inhibitory pathways have been studied and prime amongst these is NO, which have been well-known to cause relaxation of smooth muscle cells by activation of soluble guanylate cyclase. However, NOS and sGC inhibitors both did not have any effect on this urothelial-derived inhibition, suggesting that NO cannot be UDIF (Hawthorn et al., 2000). Other likely pathways that have been shown to induce smooth muscle relaxation including the COX activity, P2Y receptors and GABA receptors, were also rejected as UDIF because inhibition of these pathways did not alter urothelium depressant effect (Hawthorn et al., 2000).

It was shown in the urinary bladder that P2X and P2Y receptors are present and can be activated by neural and urothelial ATP to induce contractions and relaxation, respectively (Hernandez et al., 1999). While

P2Y receptors have been ruled out as the inhibitory pathway for UDIF in the bladder, ATP is broken down into adenosine by ectoATPases and nucleotidases, which are present in the surroundings of the nerve terminal (Cusack and Hourani, 1984) and this can stimulate the adenosine receptors which can affect smooth muscle contractility. Smooth muscle adenosine receptors have been shown to generally cause relaxation where all four subtypes are G-protein coupled receptors A_1 and A_3 are coupled to inhibitory G-protein while the activation of A_{2A} and A_{2B} receptors stimulates adenylyl cyclase (Burnstock and Kennedy, 1985, Collis and Hourani, 1993, Alexander et al., 1994, Fredholm et al., 1994). In a study of the porcine intravesical ureter, it was proposed that A_{2B} -receptor could be responsible for the mediation of smooth relaxation induced by NANC transmission (Hernandez et al., 1999).

The aim of this study was to determine the effect of the urothelium, and ATP, on ureteral smooth muscle contraction to α -adrenoceptor and 5-HT receptor stimulation and whether this effect is altered with age. In addition to this, we were also interested in determining the receptor through which, if any ATP effect is mediated.

5.2 Methods and Materials

Tissue preparation and *in vitro* functional studies were performed as stated in Section 2.1. For the first part of the study, isolated ureteral tissue strips were paired where one strip had intact urothelium while the other, urothelium was removed. Contractile responses to phenylephrine ($7.48 \times 10^{-8} - 7.48 \times 10^{-4}$ M) and 5-HT ($5.88 \times 10^{-8} - 5.88 \times 10^{-4}$ M) were compared in the absence and presence of urothelium. In the second part of the study, 1mM exogenous ATP was added into the organ baths containing isolated denuded ureteral strips and was incubated for 30 minutes before contractile responses to phenylephrine ($7.48 \times 10^{-8} - 7.48 \times 10^{-4}$ M) and 5-HT ($5.88 \times 10^{-8} - 5.88 \times 10^{-4}$ M) were recorded. These first two sets of experiments were performed in tissues from both age group: young (20 weeks) and old (56 weeks).

In the third part of this study, we utilised adenosine receptor antagonist 8-phenyltheophylline (8-PT), the P2 receptor antagonist suramin, and ATP hydrolysis enzyme apyrase (which is responsible for ATP hydrolysis into adenosine monophosphate and inorganic phosphate). Each antagonist was incubated with denuded tissue strips, together with ATP, or tissues with intact urothelium for 30 minutes before contractile responses to phenylephrine ($7.48 \times 10^{-8} - 7.48 \times 10^{-4}$ M) were recorded. In addition to this, a combination of suramin and 8-PT was incubated with denuded tissues together with exogenous ATP and intact tissue strips. Contractile responses to agonists are expressed as AUC (g s) and frequency (Hz).

Paired student's *t*-tests were performed on studies with two groups while two-way ANOVA was used for studies involving more than two groups.

5.3 Results

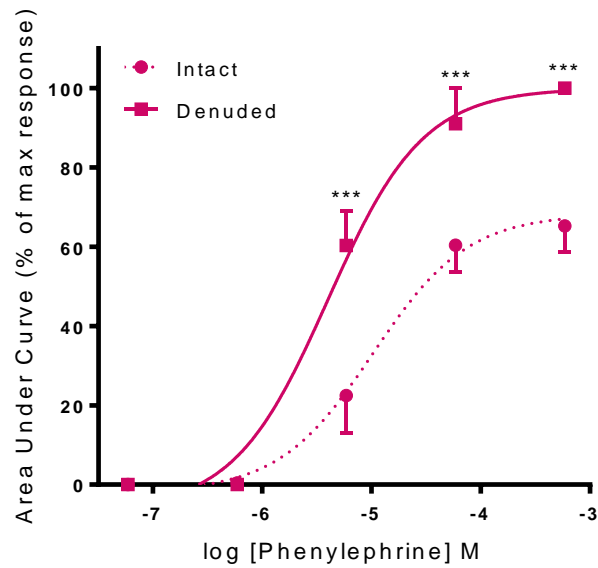
Effect of Urothelium on Agonist-Induced Ureteral Responses

Contractile responses expressed as AUC and frequency to phenylephrine were enhanced in tissues denuded of urothelium, in both age groups ($p < 0.005$, paired t -test, $n=6$, Figures 5.3 and 5.4). The inhibitory effect of the urothelium on maximal phenylephrine-induced contractile response expressed as AUC was similar in tissues from both age groups ($31.7 \pm 5.0\%$ vs $30.0 \pm 5.8\%$, young vs old). This was also observed for responses when expressed as maximum frequency of contractions ($22.3 \pm 3.6\%$ vs $27.4 \pm 5.7\%$, young vs old). The potency (pEC_{50}) of phenylephrine was not significantly different in intact and denuded tissues from younger animals ($p > 0.05$, Table 5.1). However, potency of phenylephrine was greater in the presence of urothelium in tissues from older animals ($p < 0.05$, paired t -test, $n=6$, Table 5.1).

Maximum contractility in response to 5-HT stimulation in isolated ureteral strips, expressed as AUC, was decreased in the presence of urothelium in tissues from both old and young animals ($p < 0.001$, paired t -test, $n=6$, Figures 5.5a and 5.6a). Inhibition of contractile response expressed as AUC at maximal 5-HT concentration was greater in tissues from older animals ($29.7 \pm 5.6\%$ vs $40.4 \pm 2.1\%$, young vs old, unpaired t -test, $p < 0.05$). Maximum frequency of contractions in response to 5-HT was significantly depressed only in tissues from older animals ($p < 0.001$, paired t -test, $n=6$, Figure 5.6b) and consequently, percentage of inhibition of maximum frequency was greater in older animals (9.6 ± 4.5 vs 39.3 ± 6.1 , young vs old, unpaired t -test, $p < 0.005$). Potency of 5-HT was similar in both intact and denuded smooth muscle strips in both age groups ($p > 0.05$, Table 5.1).

The degree on inhibition of contractile responses by the urothelium (AUC and frequency) was not significantly different between the two agonists, phenylephrine and 5-HT. The only observed significant difference was that in tissues from the younger animals, maximum frequency was not inhibited by the presence of urothelium in response to 5-HT but was depressed by the urothelium in response to phenylephrine.

(a)



(b)

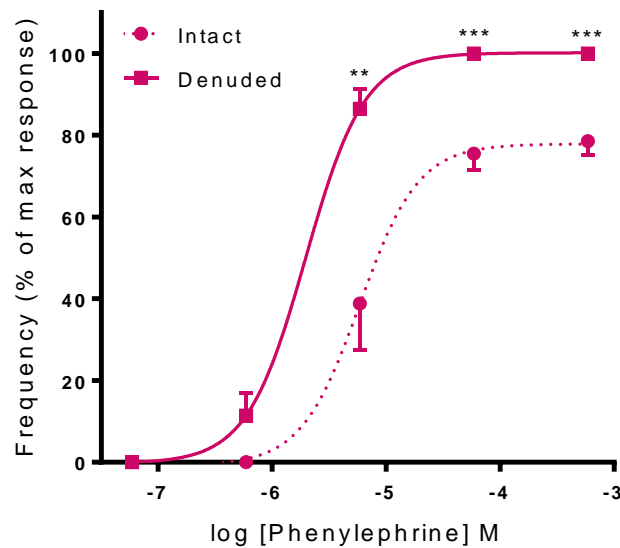
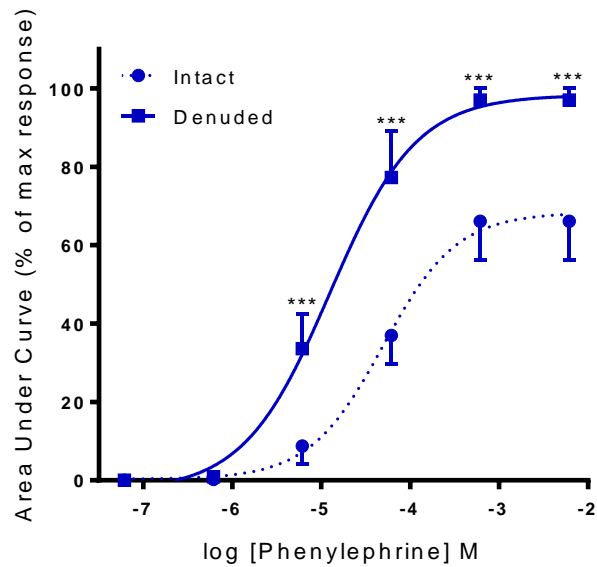


Figure 5.3 Concentration-response curves for phenylephrine in isolated distal ureter from young pigs with intact and denuded urothelium. Responses are expressed as % of denuded tissue maximal response for AUC (a) and frequency (b). Results are represented as mean \pm SEM of 6 preparations for each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs intact).

(a)



(b)

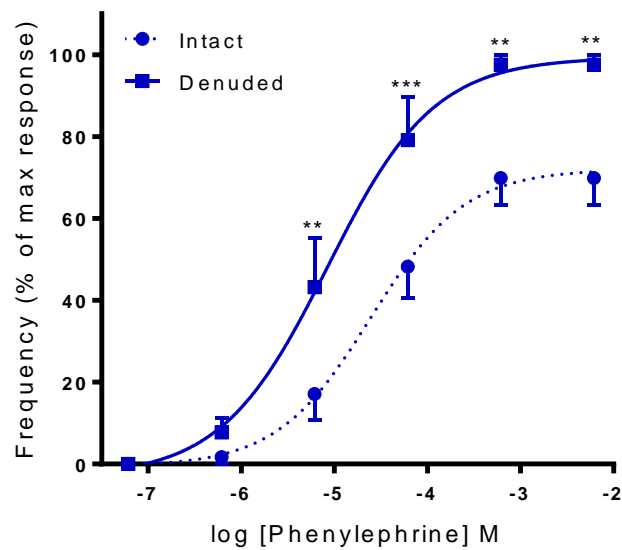
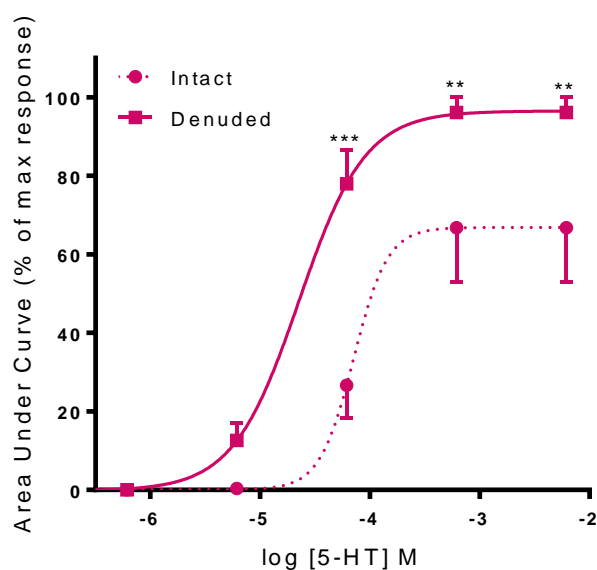


Figure 5.4 Concentration-response curves for phenylephrine in isolated distal ureter from old pigs with intact and denuded urothelium. Responses are expressed as % of denuded tissue maximal response for AUC (a) and frequency (b). Results are represented as mean \pm SEM of 6 preparations for each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs intact).

(a)



(b)

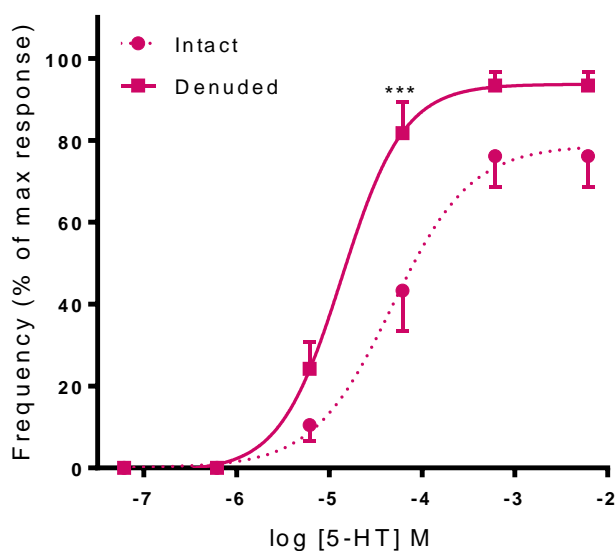
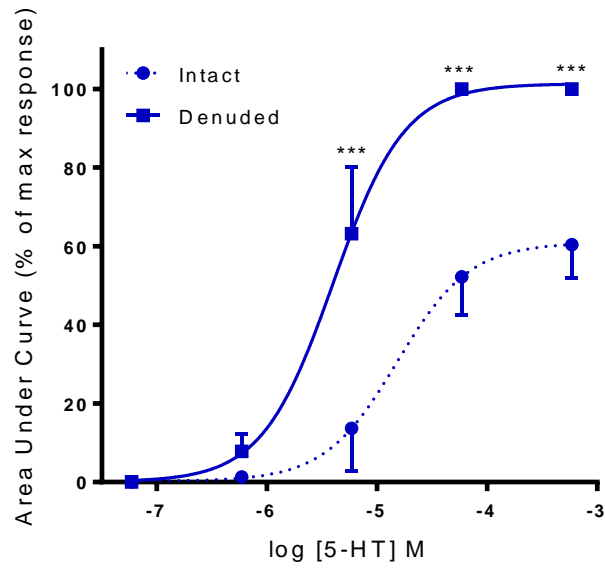


Figure 5.5 Concentration-response curves for **5-HT** in isolated distal ureter from young pigs with **intact and denuded urothelium**. Responses are expressed as % of denuded tissue maximal response for AUC (a) and frequency (b). Results are represented as mean \pm SEM of 6 preparations for each group (* p <0.05, ** p <0.01, *** p <0.001 vs intact).

(a)



(b)

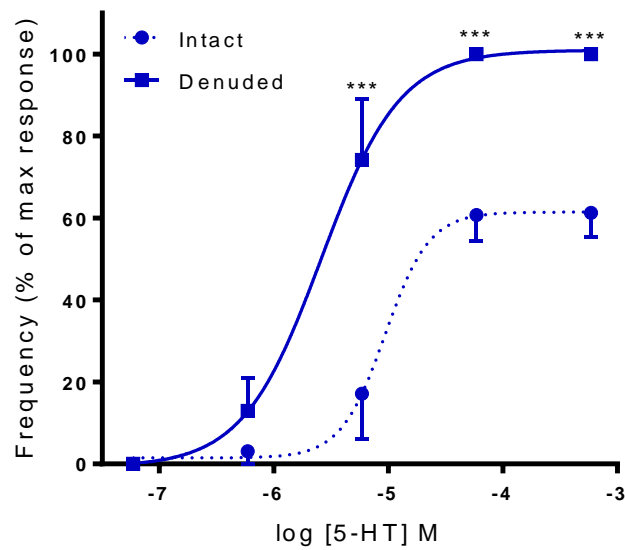


Figure 5.6 Concentration-response curves for **5-HT** in isolated distal ureter from old pigs with **intact and denuded urothelium**. Responses are expressed as % of denuded tissue maximal response for AUC (a) and frequency (b). Results are represented as mean \pm SEM of 6 preparations for each group (* p <0.05, ** p <0.01, *** p <0.001 vs intact).

Table 5.1 pEC₅₀ values for AUC for responses to phenylephrine and 5-HT in the pig distal ureter in the presence and absence of urothelium. Results are expressed as mean ± SEM, * vs intact

Agonist	Group	n	pEC ₅₀
Phenylephrine	Young	Intact	6 4.99 ± 0.19
		Denuded	6 5.40 ± 0.12
	Old	Intact	6 4.31 ± 0.19
		Denuded	6 4.90 ± 0.15*
5-HT	Young	Intact	6 4.15 ± 0.68
		Denuded	6 4.65 ± 0.09
	Old	Intact	5 4.82 ± 0.27
		Denuded	5 5.40 ± 0.15

Effect of ATP on Agonist-Induced Ureteral Responses

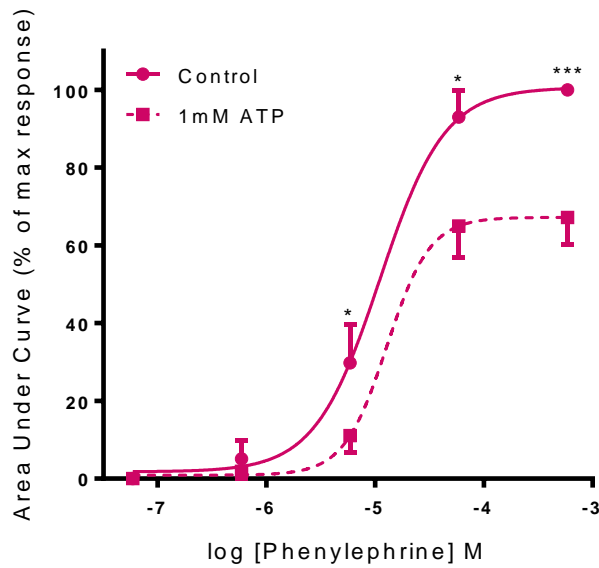
Maximum contractile responses expressed as AUC to phenylephrine were significantly reduced in the presence of ATP in denuded strips for both age groups ($p > 0.005$, paired t -test, $n=6$, Figure 5.7a and 5.8a). The inhibition induced by ATP at the maximal phenylephrine concentration was significantly greater in tissues from the older animals ($33.4 \pm 5.6\%$ vs $45.4 \pm 3.3\%$, young vs old, unpaired t -test, $p < 0.05$). Maximum frequency of contraction in response to phenylephrine was also reduced in the presence of ATP in tissues from both age groups ($p < 0.05$, Figures 5.7b and 5.8b). This inhibitory effect of ATP on maximal contraction frequency was also greater in the older animals ($22.3 \pm 4.7\%$ vs $41.7 \pm 2.8\%$, young vs old, unpaired t -test, $p < 0.005$). The potencies of both phenylephrine and

5-HT were not significantly different in the presence and absence of ATP in both age groups ($p > 0.05$, $n = 6$, Table 5.2).

Maximum contractility to 5-HT stimulation in isolated ureteral strips expressed as AUC was attenuated in the presence of ATP in tissues from both old and young animals ($p < 0.001$, paired t -test, $n = 6$, Figures 5.9a and 5.10a) and this inhibition was similar in both age groups ($22.8 \pm 4.9\%$ vs $26.3 \pm 4.7\%$, young vs old). ATP significantly depressed maximum frequency of contractions in response to 5-HT only in tissues from the older animals ($p < 0.05$, paired t -test, $n = 6$, Fig 5.10b). Hence, the percentage of inhibition on frequency response was greater in tissues from the older group ($10.9 \pm 5.4\%$ vs $23.2 \pm 4.2\%$, young vs old, unpaired t -test, $p < 0.05$).

In tissues from the younger animals, inhibition of contractions by ATP at maximal contraction expressed as AUC was similar for both agonists, phenylephrine and 5-HT ($p > 0.05$). However, maximal frequency was unaffected by ATP when stimulated with 5-HT, but was significantly decreased by ATP in phenylephrine-induced contractile responses. In the older animals, the inhibitory effect of ATP was greater on phenylephrine-induced contractile responses in comparison to 5-HT-induced responses, expressed as both AUC ($p < 0.05$, unpaired t -test) and frequency ($p < 0.005$, unpaired t -test).

(a)



(b)

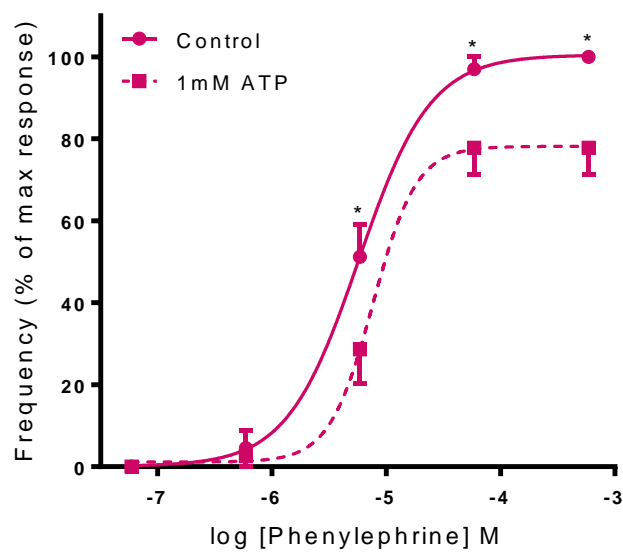
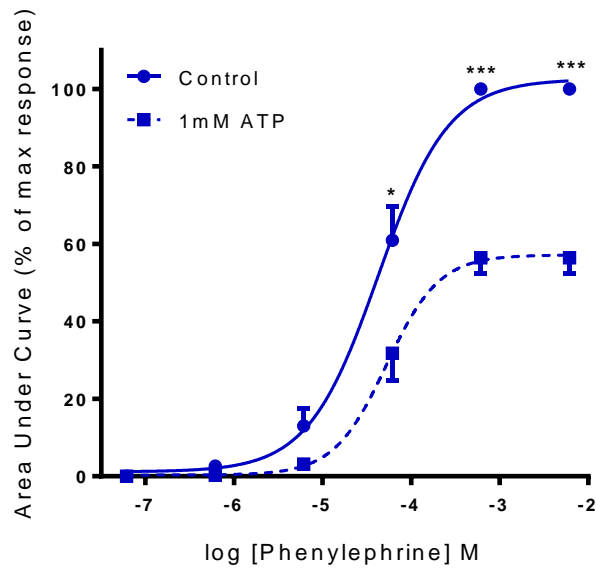


Figure 5.7 Concentration-response curves for phenylephrine in isolated distal ureter from young pigs in the presence and absence of ATP (1mM).

Responses are expressed as % of control tissue maximal response for AUC (a) and frequency (b). Results are represented as mean \pm SEM of 6 preparations for each group (* p <0.05, ** p <0.01, *** p <0.001 vs ATP).

(a)



(b)

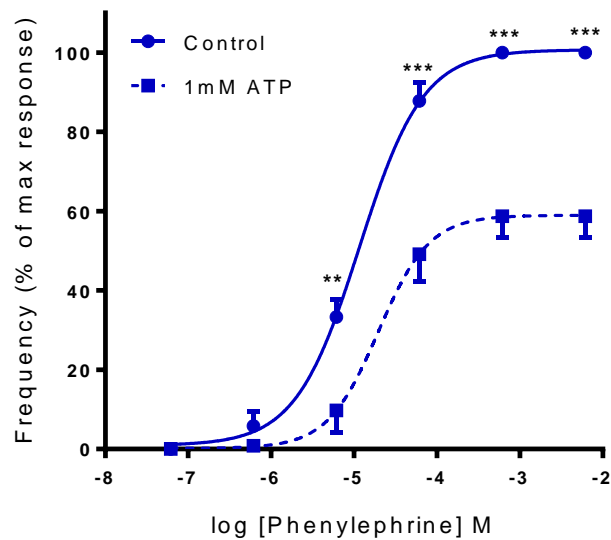
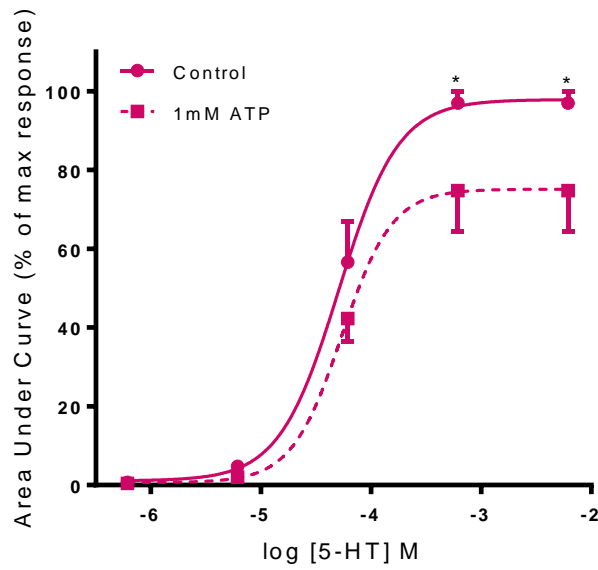


Figure 5.8 Concentration-response curves for phenylephrine in isolated distal ureter from old pigs in the presence and absence of ATP (1mM). Responses are expressed as % of control tissue maximal response for AUC (a) and frequency (b). Results are represented as mean \pm SEM of 6 preparations for each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs ATP).

(a)



(b)

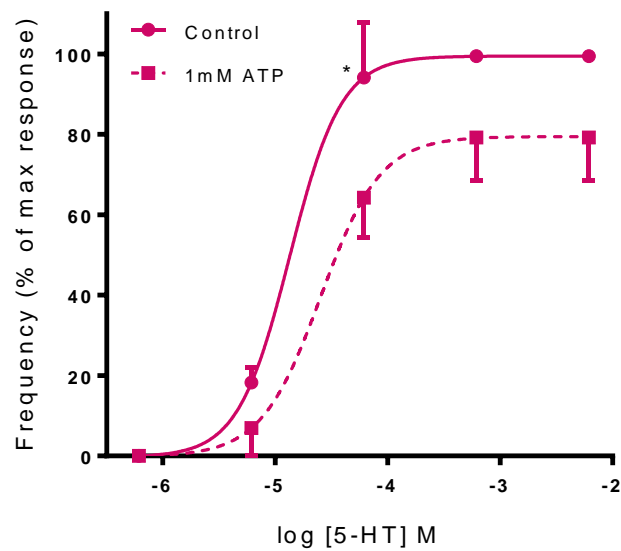
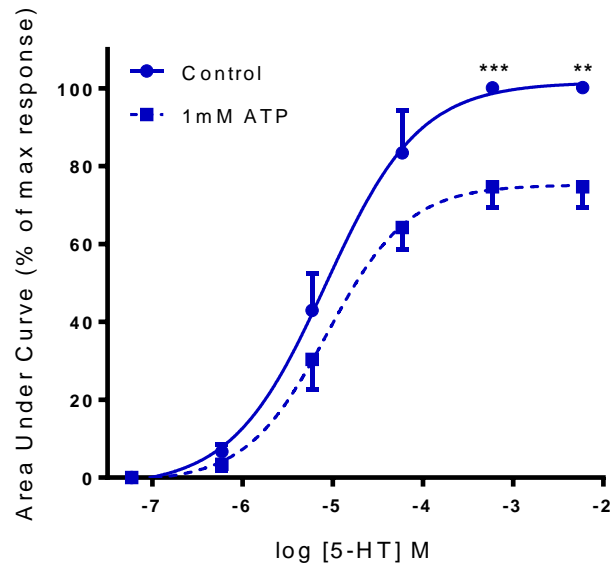


Figure 5.9 Concentration-response curves for 5-HT in isolated distal ureter from young pigs in the presence and absence of ATP (1mM). Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are represented as mean \pm SEM of 6 preparations for each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs ATP).

(a)



(b)

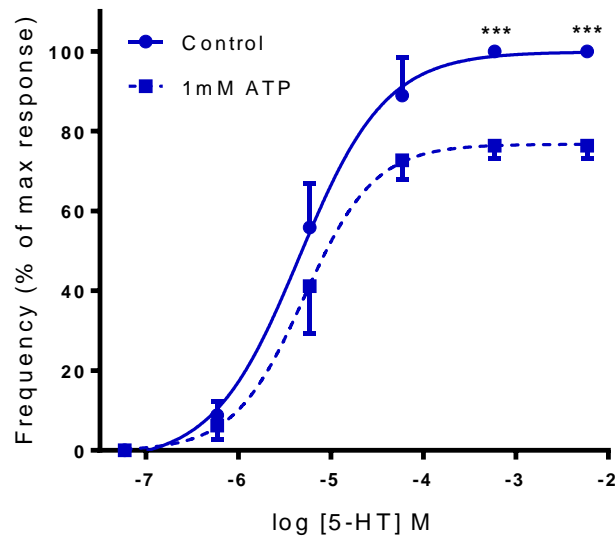


Figure 5.10 Concentration-response curves for **5-HT** in isolated distal ureter from old pigs in the presence and absence of **ATP (1mM)**. Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are represented as mean \pm SEM of 6 preparations for each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs ATP).

Table 5.2 pEC₅₀ values for AUC for responses to phenylephrine and 5-HT in the pig distal ureter in the presence and absence of ATP (1mM). Results are expressed as mean ± SEM.

Agonist	Group	n	pEC ₅₀	
Phenylephrine	Young	Control	6	4.96 ± 0.12
		ATP (1mM)	6	4.89 ± 0.24
	Old	Control	6	4.37 ± 0.08
		ATP (1mM)	6	4.28 ± 0.09
5-HT	Young	Control	6	4.29 ± 0.08
		ATP (1mM)	6	4.27 ± 0.14
	Old	Control	5	5.04 ± 1.19
		ATP (1mM)	5	5.19 ± 0.87

Comparison of Inhibitory Effect by ATP and the Urothelium

When comparing the inhibition induced by exogenous ATP and the urothelium, it was found that the inhibition was similar for both on both agonist-induced maximal contractions in the younger animals. However, in tissues from the older animals, urothelium inhibition of phenylephrine-induced contraction was lower in comparison to the inhibition by ATP, for responses expressed as both AUC and frequency (unpaired *t*-test, $p < 0.05$, Table 5.3). The opposite was demonstrated for 5-HT-stimulated maximal contractions, where the inhibitory effect of ATP was greater than the inhibitory effect of the urothelium (unpaired *t*-test, $p < 0.05$, Table 5.3).

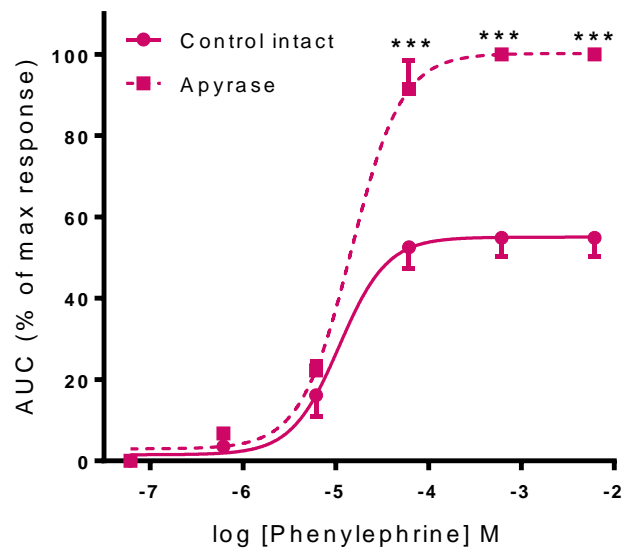
Table 5.3 Percentage of inhibition by urothelium and ATP (1mM) at maximal contractile response (expressed as AUC and frequency) to phenylephrine and 5-HT in the pig distal ureter. Results expressed as mean \pm SEM, * $p < 0.05$ vs ATP.

Agonist	Group		Urothelium (%)	ATP (%)
Phenylephrine	Young	AUC	31.7 \pm 5.0	33.4 \pm 5.6
		Frequency	22.3 \pm 3.6	22.3 \pm 4.7
	Old	AUC	30.0 \pm 5.8*	45.4 \pm 3.3
		Frequency	27.4 \pm 5.7*	41.7 \pm 2.8
5-HT	Young	AUC	29.7 \pm 5.6	22.8 \pm 4.9
		Frequency	9.6 \pm 4.5	10.9 \pm 5.4
	Old	AUC	40.4 \pm 2.1*	26.3 \pm 4.7
		Frequency	39.3 \pm 6.1*	23.2 \pm 4.2

Effect of Apyrase and ATP Receptor Antagonists on Phenylephrine-Induced Ureteral Responses in Young Animals

Apyrase (2u/ml) enhanced phenylephrine-induced contractile responses, expressed as both AUC and frequency, in intact isolated distal ureteral strips from younger animals (paired *t*-test, $p < 0.005$, Figure 5.11). This excitatory effect of apyrase on contraction to phenylephrine was not observed in tissues that were denuded of urothelium ($p > 0.05$, Figure 5.12). The inhibition of phenylephrine-induced contractile responses of denuded tissues by pre-incubation with ATP (1mM) was nullified in the presence of apyrase and this was observed for both AUC and frequency of contraction (paired *t*-test, $p < 0.001$, Figure 5.13). There was no significant difference in contractile responses in the presence of suramin (100 μ M) and/or 8-phenyltheophylline (10 μ M) in intact or denuded tissues and in the presence and absence of ATP (Figures 5.14, 5.15 and 5.16). The potency (pEC_{50}) of phenylephrine was similar in these studies (Table 5.4 and 5.5).

(a)



(b)

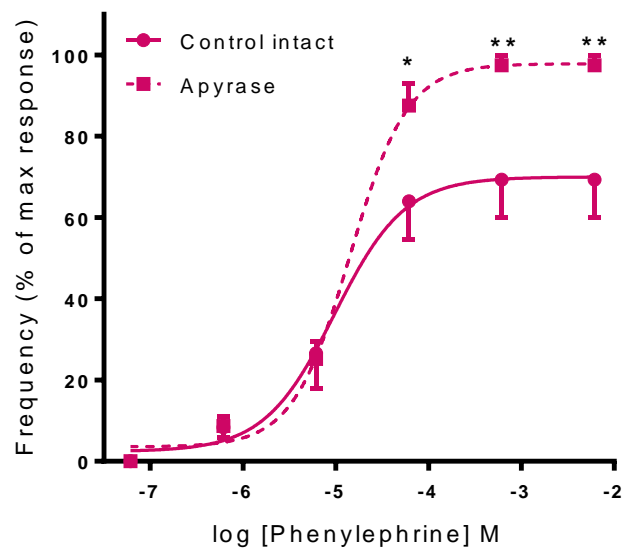
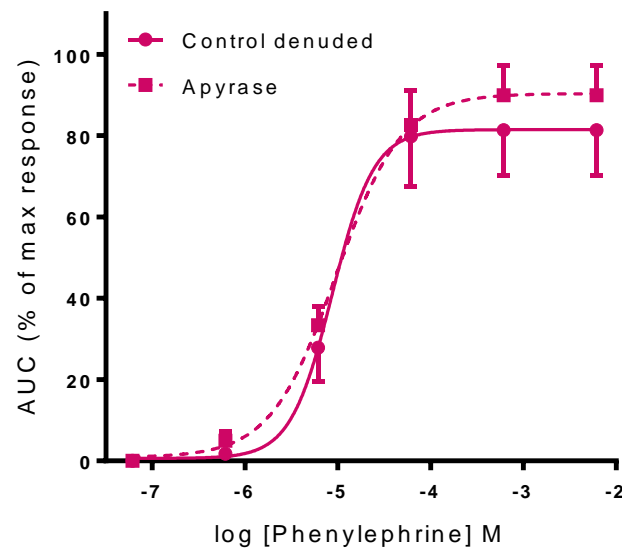


Figure 5.11 Concentration-response curves for phenylephrine in isolated distal ureter (intact) from young pigs in the presence and absence of apyrase (2units/ml). Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are represented as mean \pm SEM of 6 preparations for each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs apyrase).

(a)



(b)

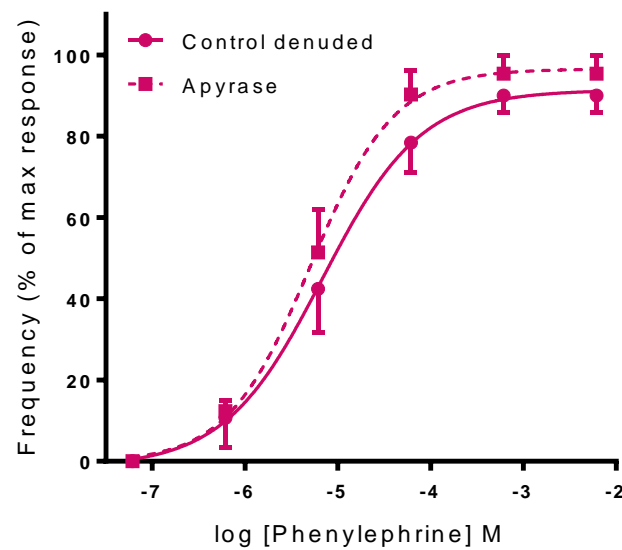
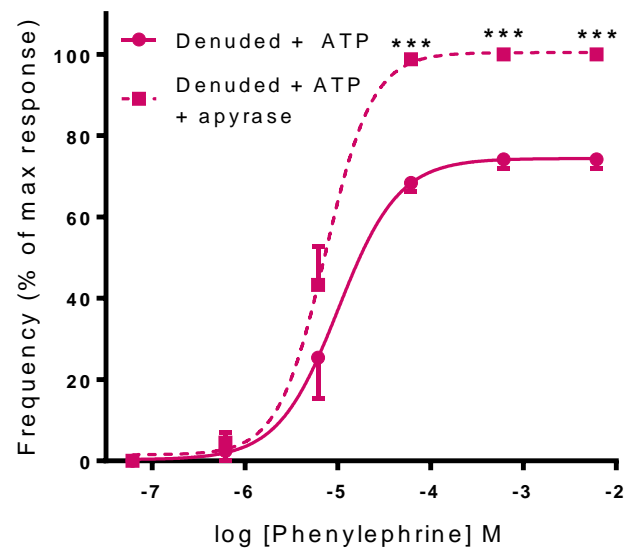


Figure 5.12 Concentration-response curves for phenylephrine in isolated distal ureter (**denuded**) from young pigs in the presence and absence of **apyrase (2units/ml)**. Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are represented as mean \pm SEM of 6 preparations for each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs apyrase).

(a)



(b)

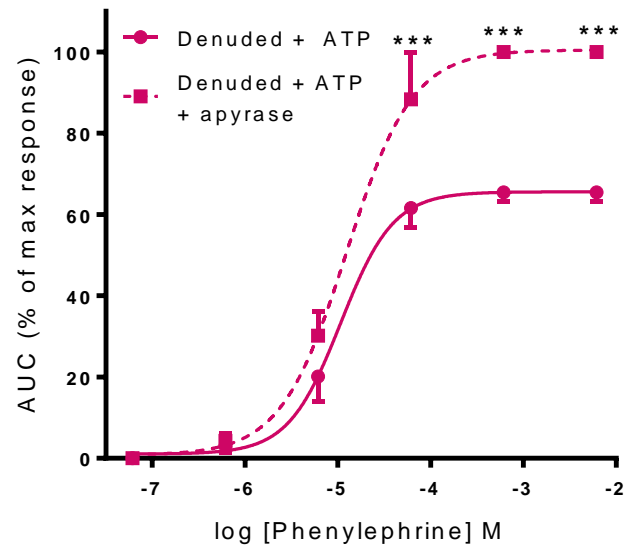
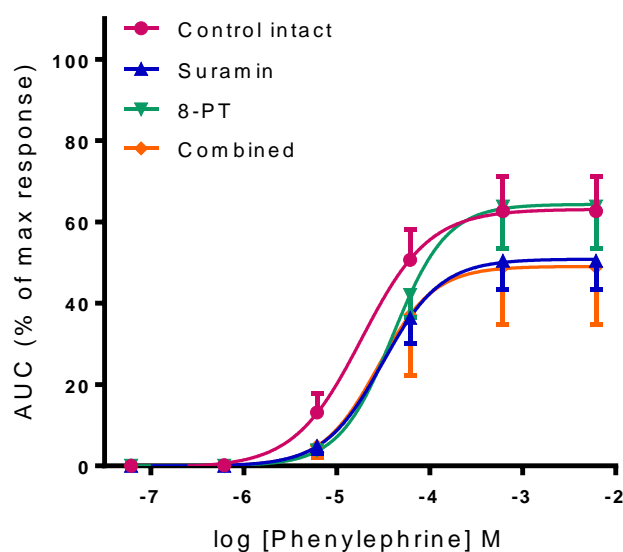


Figure 5.13 Concentration-response curves for phenylephrine with ATP (1mM) in isolated distal ureter (denuded) from young pigs in the presence and absence of apyrase (2units/ml). Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are represented as mean \pm SEM of 6 preparations for each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs apyrase).

Table 5.4 *pEC₅₀ values for AUC for contractile responses to phenylephrine in the pig distal ureter in the presence and absence of apyrase (2u/ml). Results are expressed as mean ± SEM.*

Group		n	pEC ₅₀
Intact	Control	6	4.92 ± 0.15
	Apyrase	6	4.84 ± 0.06
Denuded	Control	6	5.06 ± 0.22
	Apyrase	6	5.02 ± 0.12
Denuded + ATP (1mM)	Control	5	4.97 ± 0.10
	Apyrase	5	4.91 ± 0.10

(a)



(b)

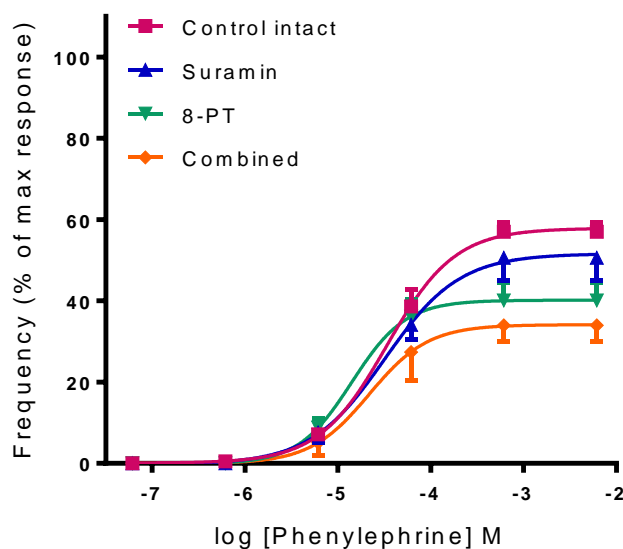
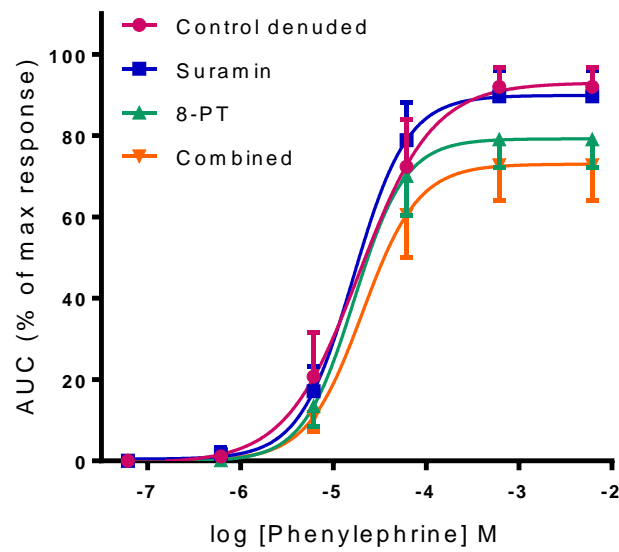


Figure 5.14 Concentration-response curves for phenylephrine in isolated distal ureter (intact) from young pigs in the presence and absence of suramin (100 μ M) and/or 8-phenyltheophylline (10 μ M). Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are represented as mean \pm SEM of 5 preparations for each group (* p <0.05, ** p <0.01, *** p <0.001 vs apyrase).

(a)



(b)

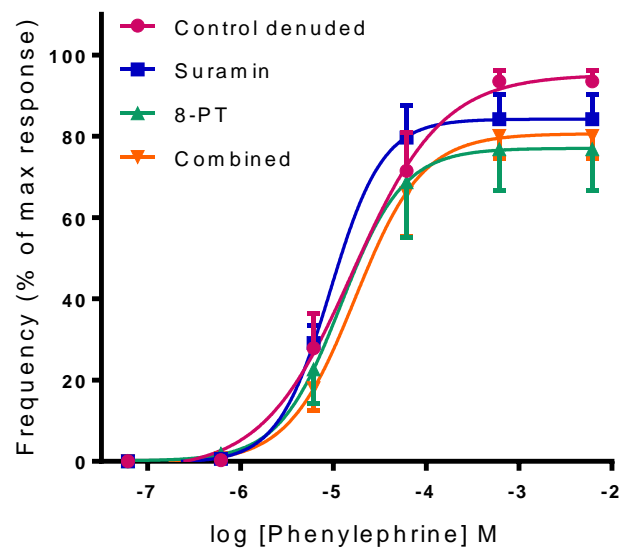


Figure 5.15 Concentration-response curves for phenylephrine in isolated distal ureter (**denuded**) from young pigs in the presence and absence of **suramin (100 μ M)** and/or **8-phenyltheophylline (10 μ M)**. Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are represented as mean \pm SEM of 5 preparations for each group (* p <0.05, ** p <0.01, *** p <0.001 vs apyrase).

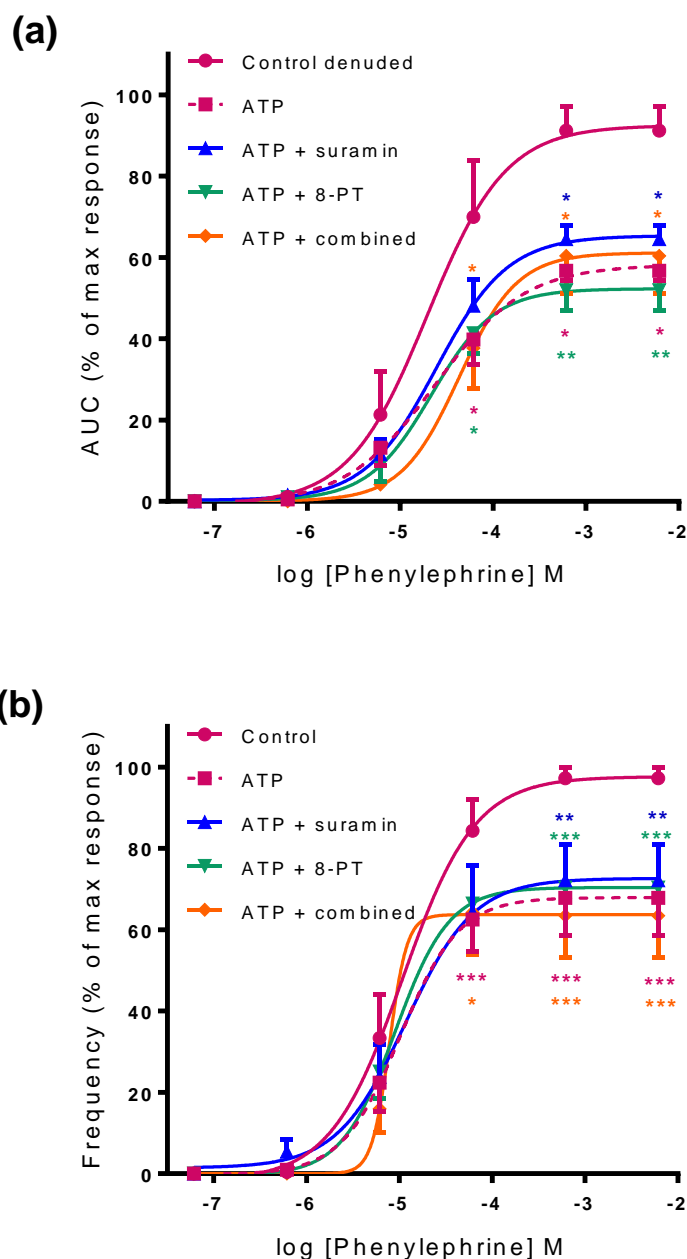


Figure 5.16 Concentration-response curves for phenylephrine with ATP (1mM) in isolated distal ureter (denuded) from young pigs in the presence and absence of suramin (100μM) and/or 8-phenyltheophylline (10μM).

Responses are expressed as % of maximal response for AUC (a) and frequency (b). Results are represented as mean \pm SEM of 5 preparations for each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs apyrase).

Table 5.5 pEC₅₀ values for AUC for contractile responses to phenylephrine in the pig distal ureter in the presence and absence of suramin (100μM) and 8-phenyltheophylline (100μM). Results are expressed as mean ± SEM.

Group		n	pEC ₅₀
Intact	Control	5	4.73 ± 0.19
	Suramin	5	4.51 ± 0.18
	8-PT	5	4.40 ± 0.17
	Combination	5	4.55 ± 0.39
Denuded	Control	5	4.72 ± 0.16
	Suramin	5	4.79 ± 0.13
	8-PT	5	4.78 ± 0.15
	Combination	5	4.69 ± 0.18
Denuded + ATP (1mM)	Control	5	4.62 ± 0.14
	Suramin	5	4.61 ± 0.11
	8-PT	5	4.63 ± 0.15
	Apyrase	5	4.36 ± 0.18

5.4 Discussion

Presence of Urothelium-Derived Inhibitory Factor in Porcine Distal Ureter

There is limited research on the urothelium of the ureter, and therefore it is tempting to speculate on the basic functionality and morphology of the urothelium in the ureter based on studies in the bladder, especially the distal ureter as it extends from the bladder. In the bladder, the stimulation of muscarinic receptors releases a factor from the urothelium that inhibits the underlying smooth muscle. This is supported by studies showing detrusor contractile responses to muscarinic receptor stimulation being enhanced upon removal of the urothelium (Hawthorn et al., 2000, Levin et al., 1995). Hawthorne et al. (2000) showed the release of a diffusible inhibitory agent by the urothelium, which is currently labelled the urothelium-derived inhibitory factor (UDIF). UDIF has also been shown following stimulation of α -adrenoceptors, but not histamine receptor (Templeman et al., 2002). Based on our previous functional studies in the porcine distal ureter (Chapter 2), the α -adrenergic and 5-HT system appear to play the major roles in the control of ureteral smooth muscle contraction thus were the focus of this study. Our findings show enhanced phenylephrine and 5-HT-induced contractile responses upon removal of the urothelium, which confirms the hypothesis that, similar to the bladder, the urothelium of the ureter significantly modulates contractility.

These results also support findings from a previous study in the rat ureter, where contractility was shown to be urothelium-dependently inhibited (Mastrangelo and Iselin, 2007). However, one disparity between our results and the latter study was that spontaneous contractions were also enhanced upon urothelium removal in the rat ureter, whilst in our study, development of spontaneous activity was unaltered in the porcine ureter.

Due to tachyphylaxis, none of these experiments were conducted in the same preparation and, therefore, the percentage of tissues that exhibit spontaneous contractions were always compared between two separate denuded and intact tissues. These were similar between the two groups (15% denuded vs 17% intact). Although the authors did not specify the region of ureter utilised, none of the rat ureteral strips with intact urothelium developed spontaneous activity, leading to the speculation that it was not the proximal ureter and was perhaps more likely mid or distal ureter. Thus, the contrasting results are perhaps not due to regional differences. We suspect that the most likely explanation behind this is simply differences between species.

Our results also suggest that the inhibitory effect of the urothelium was similar for both α -adrenoceptor and 5-HT receptor stimulation. Interestingly, in the rat ureter, stimulation of smooth muscle strips by norepinephrine did not induce any contractions in both intact and urothelium-free preparations, even in the presence of β -adrenergic system inhibition (Mastrangelo and Iselin, 2007). This is contradictory to our results where the presence of the urothelium produced a significant reduction in contractile responses to α -adrenoceptor stimulation, as expected since α -adrenoceptor agonists are generally known as the classic group of contractile agents in the ureteral smooth muscle, in several species including rat, porcine and human (Lang et al., 2002). Additionally, a separate study also did not detect any modification of 5-HT stimulated contractile responses upon removal of urothelium in the isolated porcine intravesical ureter (Hernandez et al., 2003). Therefore, to our knowledge, we are the first to demonstrate the release of an inhibitory factor from the urothelium upon α -adrenoceptor and 5-HT receptor stimulation in the porcine distal ureter. In the bladder, this has been termed UDIF, as it is as yet unidentified. We attempted to investigate whether ATP is the inhibitory factor in ureter.

Effect of ATP on Agonist-Induced Contractile Responses

Since there is much evidence for urothelial ATP release, the autocrine and paracrine effects of ATP have been thoroughly researched in the bladder of various species. However, similar to many other mechanisms, very little research on the effect of ATP has been directed to ureter. UDIF has been shown not to be ATP in the bladder, but there are studies on the effect of purines on ureteral smooth muscle receptors in the ureter (Hernandez et al., 1999).

In the detrusor smooth muscle, it has been identified that ATP can induce both relaxation and contraction, via P2Y and P2X receptors respectively, but the latter is reported to play a more significant role by majority of studies (Santoso et al., 2010). Also in the detrusor muscle, the relaxant effect of ATP was demonstrated in urothelium-denuded strips (McMurray et al., 1998). In the present study, we have shown for the first time, a functional effect of ATP on phenylephrine- and 5-HT-induced contractions in isolated porcine distal ureteral smooth muscle without urothelium, suggesting that ATP exerts an inhibitory effect directly on the ureteral smooth muscle strips. While it is appealing to speculate that this relaxatory effect of ATP is definitely attributed to P2Y receptors, it is vital to be aware that local factors, in particular breakdown by nucleotidases into adenosine, could be involved in the regulation of smooth muscle contraction and therefore, we further investigated this.

Comparison of Inhibition by Urothelium-Derived Inhibitory Factor and ATP

When comparing between the inhibition induced by ATP and that induced by the urothelium in tissues from the younger animals, it appears that the presence of ATP renders the ability of denuded strips to respond similarly to phenylephrine and 5-HT as if the urothelium was intact (ie. a similar

inhibition was seen between ATP and presence of the urothelium). The ATP concentration that was used was sufficient to make up for the loss of inhibition by the urothelium. This supports the idea that ATP may be the inhibitory factor released from the urothelium.

Control of ATP release from the urothelium in the bladder is well established, and several agents are involved (Andersson, 2015). Stimulation of muscarinic receptors M_1 , M_2 and M_3 in the urothelium were able to trigger urothelial release of ATP (Kullmann et al., 2008). This was further explored in a separate study where it was found that carbachol and an M_2 receptor preferential agonist oxtemorine stimulated ATP release. This release was antagonised by methoctramine, an M_2 receptor selective antagonist, suggesting that this muscarinic receptor subtype plays a significant role in neurotransmitter-induced ATP release from the bladder mucosa (Sui et al., 2014). This is in similarity to UDIF release, where stimulation of muscarinic receptor and α -adrenoceptor cause release of UDIF from the urothelium. While receptor expression on the urothelium of the ureter has not yet been investigated, it is possible that the stimulation of the urothelium by major neurotransmitters of the ureter (α -adrenoceptor agonists and 5-HT) could induce release of ATP from the urothelium. Although this is purely a speculation and still requires further inquisition, it provides further weight to the suggestion that ATP might be UDIF in the ureter. It is also important to keep in mind that the stimulation of P2Y receptors by ATP in the urothelium represents an important feedback mechanism to amplify further ATP release (Sui et al., 2014). Therefore, besides intrinsic ATP release from the urothelium, exogenous ATP has also been suggested to be able to stimulate the release of more ATP from the urothelium which in turn could produce more inhibition of smooth muscle contraction (Hawthorn et al., 2000, Levin et al., 1995).

In the older animals, it was demonstrated that the inhibition by ATP was greater than that induced by the urothelium for α -adrenoceptor-stimulated

contractions and vice versa for 5-HT-stimulated contractions. There have been many reports on the changes that urothelium undergoes with ageing, reflecting the ageing process and the fact that older individuals are more prone to bladder disorders (Nelson and Dannefer, 1992, Caremel et al., 2010). Some noteworthy changes with ageing that have been exhibited in the bladder urothelium are a significant increase in urothelial ATP release (Sui et al., 2014) and increased sensitivity to ATP and enhanced expression of P2X receptor (Daly et al., 2014). In addition to this, contractility in response to ATP in the aged rat bladder was also found to be significantly greater than in younger rats (Saito et al., 1993). Therefore, we suggest that findings we have presented here for ureters from older animals may be a result there could also be alterations that occur with ageing in urothelial mediator release. This supports a change in factors released from urothelium with age and could not be solely ATP.

Effect of Apyrase and Purinergic Antagonists on Urothelium-Derived Inhibitory Factor and ATP Inhibitory Effect

Due to the differences found between the inhibition induced by ATP and UDIF in the tissues from older animals, our subsequent experiments were performed only in the younger animals. We used apyrase to promote the breakdown of ATP into adenosine monophosphate and inorganic phosphate. It was observed that the presence of apyrase significantly enhanced phenylephrine-induced contractions in isolated tissues with intact urothelium, and that there was no effect of apyrase on ureteral tissues denuded of urothelium. Neither suramin nor 8-PT was able to produce a similar effect. Additionally, the inhibitory effect of presence of ATP on phenylephrine-induced contraction in denuded tissues was also reversed by apyrase but not by suramin and/or 8-PT. These findings suggest the possibility of urothelial release of ATP which inhibits smooth

muscle contractions through a mechanism other than those blocked by suramin and/or 8-PT.

The use of suramin was proven to be ineffective in diminishing the inhibitory effect of UDIF or ATP in the ureteral tissues. Although, there are currently no reports demonstrating the expression of P2X and P2Y receptors on the ureter, this expression has been shown for both the urothelium and smooth muscle of the bladder (Birder and de Groat, 2007, Sui et al., 2014). Suramin is a non-selective P2 receptor antagonist and inhibits both P2X and P2Y receptors at the same time. However, these receptors are responsible for contraction and relaxation respectively and thus, simultaneous inhibition could prove a complication in the present study.

The agonist that was used in this part of the study was phenylephrine, which is thought to induce smooth muscle contraction via activation of phospholipase C, and subsequently, IP₃-induced Ca²⁺ release from the sarcoplasmic reticulum. Previous findings have shown that ATP exerts an inhibitory effect on muscarinic-mediated contractions in the distal colon via inhibition of intracellular Ca²⁺ release (Hurley et al., 1993, Jorgensen et al., 1995, Soslau et al., 1995, Fukushi, 1999, MacMillan et al., 2012). While the contractions developed in the ureteral tissues in our studies were α -adrenoceptor-mediated responses, it is proposed that the phospholipase C pathway involved is similar to muscarinic stimulation. In the distal colon, the mechanism through which ATP induced its inhibitory effect was further examined and it was suggested that ATP is capable of suppressing IP₃-evoked Ca²⁺ in the distal colon (MacMillan et al., 2012). This suppression was shown to not involve activation of phospholipase C but via the activation G-protein-coupled receptor, P2Y₁ (MacMillan et al., 2012). Thus, we suggest the prospect of ATP release by the urothelium in the ureteral tissues which induces inhibition of contractile responses via suppression of IP₃-evoked intracellular Ca²⁺ release.

5.5 Conclusion

The identification of urothelium-derived inhibitory factor (UDIF) may provide a new target for pharmacologically mediated therapeutic intervention in the lower urinary tract, including the distal ureter where ureteral calculi frequently lodge. Since its discovery, it has proven a challenge to establish the identity of UDIF. In this study, we were able to identify ATP as a possible candidate for UDIF in isolated ureter from the younger animals. While the mechanism by which this occurs still needs to be studied in more detail, we propose that urothelially-released ATP inhibits the contractile response via inhibition of IP₃-evoked Ca²⁺ release. In addition, we showed that with age, UDIF is altered and could involve more than just ATP. While further studies are still required, especially with age, we deem these findings significant, as they provide an insight into UDIF.

CHAPTER 6:

General discussion

6

6.1 General Discussion

Aim

The overall aim of this project was to further elucidate the mechanisms controlling ureteral motility by filling in the gaps in the current research on the pharmacology and physiology of the ureter. This objective was undertaken with a purpose to identify novel targets for development of suitable drugs for medical expulsive treatment of ureteral calculi. Medical expulsive treatment can be administered to patients with smaller calculi and also as an adjunct to less invasive treatment modalities. Successful medical expulsive treatment is achieved when ureteral colic is alleviated and the rate of stone passage through the ureter is increased.

While the pathophysiology of renal colic is not completely understood, it is thought that increased intraluminal pressure within the ureteric tube significantly amplifies pain (Rose and Gillenwater, 1973). The ureter is made up of smooth muscle cells that can contract or relax on various stimulations. This is most likely the main mechanism controlling changes in intraluminal pressure. Therefore, this thesis was focused on the investigation of mechanisms involved in ureteral smooth muscle contraction.

Experimental Model

Due to the inability to obtain a sufficient number of human ureteral tissues, the experimental model that was utilised in this thesis was the porcine ureter. In *in vitro* studies, porcine tissues are frequently used as a model of human tissues. While comparison between human and porcine isolated ureters have not been specifically performed, multiple studies have reported the similarities between the porcine and human with respect to

the physiology and anatomy of the lower urinary tract. These include resemblance in urodynamic and structural characteristics, and neural control (Sibley, 1984, Crowe and Burnstock, 1989, Parsons et al., 2012). By optimization of the methodology, we were able to achieve a standard protocol that was utilised throughout all studies of this thesis. Tissue strips were suspended longitudinally due to the likelihood of both circular and longitudinal smooth muscle cells of the ureter being involved in normal ureteral peristalsis. Besides that, distal ureteral strips were isolated as samples because calculi are most frequently lodged at the distal region (El-Barky et al., 2014). By considering these factors, we ensured that the most relevant set-up was used.

Despite its resemblance with human tissues, there are limitations involved with the porcine model. Influences on the whole system associated with organ blood flow are not represented in the *in vitro* model. Therefore, responses involving the immune system and other systems upon addition of the various drugs are not mimicked in this model. In addition to this, the viability of isolated ureteral tissues is limited. This resulted in the inability to reproduce concentration response curves and prevented investigation of repeated treatments and recovery following drug incubation. However, the findings from this thesis have allowed for further investigations with other experimental methods that might fulfil the weaknesses the porcine model possesses like *in vivo* settings and eventually, clinical trials.

Ageing and Urolithiasis

There are many risk factors associated with urolithiasis including gender, race, familial history and other modifiable risk factors including diet and hydration (Gottlieb, 2002, Romero et al., 2010, Schade and Faerber, 2010, Macneil and Bariol, 2011, Nowfar et al., 2011). In this thesis, the main focus was age as a risk factor (Hess, 2003) due to differences that were observed between contractile responses in isolated ureter from younger

and older animals. It appears that in older animals, larger contractile responses are developed during α_1 -adrenoceptor and muscarinic receptor stimulation, whilst contractions to 5-HT receptor stimulation are decreased with age.

It is suggested that the differences in contractile responses vary with different systems. During stimulation of α_1 -adrenoceptors, upregulation of the specific intracellular signalling pathway might be the cause for the differences in contractile responses with age. In contrast, the difference in contractile responses to stimulation of 5-HT receptors observed with age appears to be due to the involvement of different receptor subtypes with age, as was shown in Chapter 3, where pharmacological characterization of functional 5-HT receptor subtype/s was performed. The findings suggest that the 5-HT_{2A} receptor is solely responsible for 5-HT-mediated contractile responses in the isolated ureter from younger animals. This receptor subtype also plays a mediatory role in tissues from the older animals, but one or more relaxatory 5-HT receptor subtype/s is involved with age. Hence, this could explain the decreased contractile responses to 5-HT receptor stimulation with age. Contractile responses to muscarinic responses only developed in isolated ureteral tissues from older animals, suggesting that perhaps muscarinic receptors are expressed later with age. However, further molecular studies are required to confirm this.

While nifedipine appears to be the less popular option for medical expulsive therapy, due to its various side effects, it is nevertheless a clinical option and has proven to be effective in increasing stone expulsion rate (Dellabella et al., 2005, Micali et al., 2007). This leads to the possibility of targeting other intracellular signalling pathways that might have a role in controlling ureteral smooth muscle contraction. This thesis was successful in showing the importance of the calcium sensitization pathway and Rho-kinase in the mediation of contractility of the ureter (Chapter 4). It was observed that the Rho-kinase pathway plays a

significant role in both α_1 -adrenoceptor and 5-HT receptor-stimulated ureteral responses, regardless of age. In addition to that, it was also observed that Rho-kinase activity is constant in the presence or absence of agonist (phenylephrine or 5-HT). Age-related differences in the control of Rho-kinase on frequency of phasic contractions were also observed which could be further investigated. This confirms that ROCK activity might alter with age in the lower urinary tract as previously reported (Gomez-Pinilla et al., 2011, Kirschstein et al., 2014).

Lastly, it was also shown that the urothelium has a role in the ureter and changes in the role of urothelium might occur with age. While the urothelium is usually discussed in the context of the bladder (Hawthorn et al., 2000, Murakami et al., 2007), we hypothesised that it could also play a role as important in the ureter. This hypothesis was proven correct as the urothelium-derived inhibitory factor (UDIF) that has been reported in the bladder (Hawthorn et al., 2000, Murakami et al., 2007) was also detected in the porcine distal ureter. However, there are no current report comparing the morphology of urothelium between the distal ureter and bladder and this can be further investigated in the future. More importantly, we were able to identify ATP as a possible candidate for UDIF, which previous reports have not been able to determine. Our findings also show that ATP might be solely responsible for UDIF effects in younger animals and that this system might be more complicated with age.

Concluding Remarks

Although ageing may not be the most important risk factor associated with ureteral calculi, the findings of this thesis clearly indicate the significance of ageing on mechanisms controlling ureteral motility and that these need to be taken into account when undertaking basic studies of ureteral function. While further investigations with *in vivo* studies and human tissues are still required, these findings contribute to the literature on

physiology and pharmacology of the ureter and may aid in the discovery of novel targets for medical expulsive therapy.

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APPENDICES

Appendix 1

AREA UNDER CURVE MEASUREMENT FOR LABCHART SOFTWARE

(measures overall activity of tissue strips)

1. On the Lab Chart trace, add a new Channel (eg. Channel 5) with the following settings:
Source Channel > Raw Data (eg. Channel 1, Channel 2, etc)
Calculation > Integral
Reset > No Reset
2. Label the start of baseline activity before adding drug (eg. 0.1ug baseline). * Label the start of activity after adding drug (eg. 0.1ug data).
3. Open Data Pad. On a vacant column, set the following calculations:
Statistics > End Value - Start Value
Calculation Source > Integral Channel, *ie. the channel added at Step 1* (eg. Channel 5)
4. Under Commands, select Multiple Add to Data Pad with the following settings:
Find Using > Comment
Channel > Any
Containing > Label from Step 2 (eg. 0.1ug data, 0.1ug baseline)
Select > for > 3 minutes > after comment
Step through > Whole file
5. The values added to Data Pad after the step before is the area under curve for the areas selected (3 minutes before or after adding drug). To calculate the change in activity:
$$\text{Area under curve 3 minutes after adding drug} - \text{Area under curve 3 minutes before adding drug}$$

* For single dose response, every drug concentration has a different baseline. For cumulative dose response, the same baseline (before first dose was added) is used for all concentrations.

Appendix 2

Product Manual

96-well ROCK Activity Assay Kit

Catalog Number

STA-416	96 assays
STA-416-5	5 x 96 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures



Kit Components

Box 1 (shipped at room temperature)

1. ROCK Substrate Coated Plate (Part No. 241601): One strip well 96-well plate precoated with recombinant MYPT1.
2. 10X Kinase Buffer (Part No. 241602): One bottle – 20 mL of 250 mM Tris, pH 7.5, 100 mM $MgCl_2$, 50 mM Glycerol-2-Phosphate, 1 mM Na_3VO_4 .
3. ATP Solution (Part No. 241604): One vial – 100 μ L of 100 mM ATP.
4. Anti-phospho-MYPT1 (Thr⁶⁹⁶) (Part No. 241603): One vial – 20 μ L
5. Secondary Antibody, HRP Conjugate (Part No. 231003): One vial – 20 μ L
6. Assay Diluent (Part No. 310804): One 50 mL bottle.
7. 10X Wash Buffer (Part No. 310806): One 100 mL bottle.
8. Substrate Solution (Part No. 310807): One 12 mL amber bottle.
9. Stop Solution (Part No. 310808): One 12 mL bottle.

Box 2 (shipped on blue ice packs)

1. Active ROCK-II (Part No. 241505): One vial – 20 μ L containing 10 ng active ROCK-II in 25 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5 mM Glycerol-2-Phosphate, 0.1 mM Na_3VO_4 , 10% Glycerol, 0.1% BSA.

Materials Not Supplied

1. ROCK sample (purified kinase, cell or tissue lysate)
2. Lysis Buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM 2-glycerophosphate, 1 % Triton X-100 or 1 % Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4 and Proteinase inhibitors.
3. DTT
4. 0.5 M EDTA
5. 30°C incubator or water bath
6. 10 μ L to 1000 μ L adjustable single channel micropipettes with disposable tips
7. 50 μ L to 300 μ L adjustable multichannel micropipette with disposable tips
8. Multichannel micropipette reservoir
9. Microplate reader capable of reading at 450 nm (620 nm as optional reference wave length)

Storage

Store active ROCK-II at -80°C, ATP Solution at -20°C and all other kit components at 4°C until their expiration dates. Avoid multiple freeze/thaw cycles.

Preparation of Reagents

- 10X Kinase Reaction Buffer containing DTT and ATP: Just prior to usage, add DTT to a final concentration of 10 mM and ATP to a final concentration of 2 mM to the 10X Kinase Buffer. For Example, add 10 μ L of 1M DTT (not provided) and 20 μ L of 100 mM ATP solution to 970 μ L of 10X Kinase Buffer. 10X Kinase Reaction Buffer containing DTT and ATP may be stored at 4°C for short term (1-2 weeks).
- Diluted Active ROCK-II Positive Control: Just prior to usage, dilute the provided active ROCK-II (0.5 μ g/mL) to 0.02 μ g/mL with 1X Kinase Buffer. For example, add 8 μ L of the active ROCK-II and 20 μ L of 10X Kinase Buffer to 172 μ L deionized water.
- 1X Wash Buffer: Dilute the 10X Wash Buffer Concentrate to 1X with deionized water. Stir to homogeneity.
- Anti-Phospho-MYPT1 (Thr⁶⁹⁶) Antibody and HRP-Conjugated Secondary Antibody: Immediately before use dilute the anti-phospho-MYPT1 (Thr⁶⁹⁶) antibody 1:1000 and HRP-conjugated secondary antibody 1:1000 with Assay Diluent. Do not store diluted solutions.

Assay Protocol

1. Purified kinase or cell lysate sample can be used directly in the kinase assay or further diluted with 1X Kinase Buffer. Each sample should be assayed in duplicate.
2. Add 90 μ L of the diluted active ROCK-II positive control or unknown ROCK samples to the wells of the substrate plate.
3. Initiate the kinase reaction by adding 10 μ L of the 10X Kinase Reaction Buffer containing DTT and ATP. Mix well.
4. Cover with a plate cover and incubate the wells at 30°C for 30-60 minutes with gentle agitation.
5. Stop kinase reaction by flicking out the content or by adding 50 μ L of 0.5 M EDTA, pH 8.0, to each well.
6. Remove plate cover and empty wells. Wash microwell strips 3 times with 250 μ L 1X Wash Buffer per well with thorough aspiration between each wash. After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess 1X Wash Buffer.
7. Add 100 μ L of the diluted anti-phospho-MYPT1 (Thr⁶⁹⁶) antibody to each well.
8. Cover with a plate cover and incubate at room temperature for 1 hour on an orbital shaker.
9. Remove plate cover and empty wells. Wash the strip wells 3 times according to step 6 above.
10. Add 100 μ L of the diluted HRP-conjugated secondary antibody to each well.
11. Cover with a plate cover and incubate at room temperature for 1 hour on an orbital shaker.

12. Remove plate cover and empty wells. Wash microwell strips 3 times according to step 6 above. Proceed immediately to the next step.
13. Warm Substrate Solution to room temperature. Add 100 μL of Substrate Solution to each well, including the blank wells. Incubate at room temperature for 5-20 minutes on an orbital shaker.
14. Stop the enzyme reaction by adding 100 μL of Stop Solution into each well, including the blank wells. Results should be read immediately (color will fade over time).
15. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wave length.

Example of Results

The following figure demonstrates typical results seen with Cell Biolabs' 96-well ROCK Activity Assay Kit. One should use the data below for reference only.

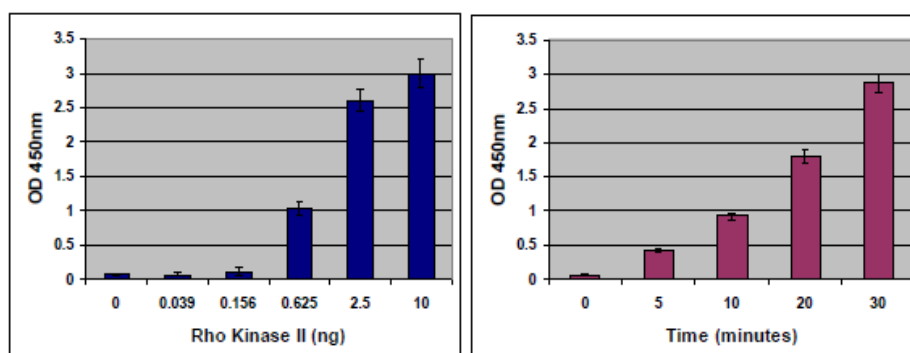


Figure 1: ROCK-II Activity Assay. Left: Active ROCK-II was incubated for 60 minutes at 30°C. Right: 2.5 ng of active ROCK-II was incubated at 30°C for times as shown. Phosphorylation of MYPT1 substrate was detected by anti-phospho-MYPT1 (Thr⁵⁹⁶) antibody as described in Assay Protocol.